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Award Number: W81XWH-08-1-0605

TITLE: The Role of Polycomb Group Gene BMI1 in the Development of Prostate Cancer

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REPORT DATE: July 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
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1. REPORT DATE (DD-MM-YYYY) July 2012		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 September 2011-6 June 2012	
4. TITLE AND SUBTITLE The Role of Polycomb Group Gene BMI1 in the Development of Prostate Cancer			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-08-1-0605		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Mohammad Saleem Bhat 'msbhat@hi.umn.edu'			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Minnesota Minneapolis, MN 55455			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S.Army Medical Research and Material Command Fort Detrick, Maryland, MD, 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: We proposed to investigate the role of BMI1 (a member of polycomb gene family) in human prostate cancer (CaP) development. Here, we present the work accomplished during the last 5 months (after submitting the 1 st annual report) of the project. In 1 st annual report we showed that BMI1 protein levels are highly elevated in human CaP patients and we investigated the mechanistic basis of the role of BMI1 in human CaP. We showed that BMI1-silenced CaP cells exhibit decreased proliferative and clonogenic potential. On the contrary, BMI1-overexpressing CaP cells exhibited the reverse. Based on the outcome of micro-array and PCR array analysis, we showed that silencing of BMI1 caused a decrease in the cyclin D1 (Wnt target) and Bcl-2 (Sonic Hedgehog-SHH target), however an increase in p16 was observed. Conversely, overexpression of BMI1 exhibited the reverse effects. In the current report we provide novel findings about the transcriptional activation of Bcl-2 in CaP cells. We provide evidence that BMI1 induces the Bcl-2 expression in CaP cells suggesting the involvement of other pathway too in the regulation of Bcl-2 transcriptional activation. Another important finding of our report is that we identified those Bcl-2 acts as a novel Wnt target in CaP cells. We found that BMI1 regulates (i) activity of TCF4 transcriptional factor and (ii) binding of TCF4 to the promoter region of anti-apoptotic <i>BCL2</i> gene. Notably, an increased TCF4 occupancy on <i>BCL2</i> gene was observed in prostatic tissues exhibiting high BMI1 levels. Using tumor cells other than CaP, we also showed that regulation of TCF4-mediated BCL2 by BMI1 is universal. It is noteworthy that forced expression of BMI1 was observed to drive normal cells to hyperproliferative mode. We show that targeting BMI1 improves the outcome of docetaxel therapy in animal models bearing chemoresistant prostatic tumors. Finally, by employing BlueGen/L Super computer modeling, we identified small molecule inhibitors of BMI1. We suggest that BMI1 could be exploited as a potential molecular target for therapeutics to treat chemoresistant tumors.					
15. SUBJECT TERMS BMI1, Wnt Signaling, Bcl-2, TCF, Prostate Cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction:

Prostate cancer (CaP) is the most common visceral cancer diagnosed in men; it is the second leading cause of cancer related deaths in males in the United States and the Western world (1). Prostate cancer (CaP) patients (30-50%) will have a local or distant recurrence of disease after surgery or radiation therapy (2-4). Although castration is a common treatment option for metastatic CaP, it does not significantly prolong the survival of patients and majority of these patients progress to castration-resistant prostate cancer (CRPC). A treatment option for CRPC is cytotoxic chemotherapy; however, chemotherapy improves overall survival in such patients by only a median of 2.9 months (4). Despite chemotherapy, CRPC patients typically show rapid progression and develop chemoresistant disease (4-5). Therefore emergence of chemoresistance is considered a major hurdle in the management of CaP. The dismal outcome of the management of chemoresistant CRPC disease could also be associated to the lack of knowledge about the molecular mechanism involved in the development of chemoresistant disease.

There is increasing evidence that polycomb group (PcG) proteins, first discovered in *Drosophila* as epigenetic gene silencers of homoeotic genes, play a crucial role in cancer development and disease recurrence (6). BMI1, a member of PcG family of repressor proteins, is a well-known marker used in stem cell biology (6-7). There is an enormous body of evidence suggesting that increased expression of BMI1 could facilitate chemoresistance in solid tumors (6-7). Recent studies show that BMI1 is positively correlated with poor prognosis in cancer patients (8-11). We recently reviewed the significance of BMI1 in the emergence of chemoresistance in various types of cancers (6). Glinsky *et al* identified BMI1 as one the signature molecules in a broad spectrum of therapy-resistant cancers included CaP (12). Except a few regulatory functions of BMI1 in cell cycle (suppressing p16INK4a and p14ARF), not much is known about the mechanism of action of BMI1. In this current study, we determined the relevance of BMI1 in the chemoresistance of CaP disease and delineate its mechanism of action both *in vitro* and *in vivo*. In addition, we establish the utility of BMI1 as a molecular target for therapeutic agents to overcome chemoresistance.

Body

Under this section we provide information about the experimental and materials and methods used to accomplish our objectives as stated in the proposal.

Experimental Design for Specific Aim #1.

We conducted the experiments to define the effect of overexpression and silencing of BMI1 gene in CaP cells. For this purpose, we (a) knockdown the BMI1 gene by transfection of siRNA and (b) overexpressed the BMI1 gene by transfecting BMI1 construct (pbabe-BMI1 plasmid provided by

Professor Chi Van Dang, Professor of Cell Biology, School of Medicine, The Johns Hopkins University, Baltimore, MD) in PC3 (androgen-independent), LNCaP (androgen-dependent), 22R \square 1 (androgen-sensitive) and normal prostate epithelial cells (PrEC) cells. We then studied the growth and viability of transfected cells *in vitro* by employing the MTT assay. To investigate the effect of BMI1 gene on the rate of proliferation of CaP cells, we employed ^3H thymidine uptake assay. This assays measures the amount of ^3H thymidine taken up by dividing cells (for DNA synthesis) thus gives a measure of the rate of division or proliferation of cells. BMI1 silenced and BMI1 overexpressing CaP cells were cultured in presence of ^3H thymidine and ^3H thymidine uptake was measured by Liquid scintillation counter. These cells were also measured for DNA content. Since BMI1 was observed to increase the proliferative potential of CaP cells and to establish that BMI1 indeed was a driving force for proliferating cells, we investigated whether BMI1 has to potential to drive proliferation of normal prostate epithelial cells. For this purpose, BMI1 was overexpressed in normal prostate epithelial cells (PrEC). We chose PrEC cells because under normal culture conditions, PrEC cells are known to replicate between 3-4 cycles and after 4 cycles, these cells enter into a mode of senescence. The break of senescence in normal epithelial cells is a hall mark of progression towards proliferation. As a control to study, another set of PrEC cells were transfected alone vector (pbabe). Further a microarray was performed with BMI1 silenced LNCaP cells to understand the mechanism of action of BMI1 in CaP cells. Experiments conducted under this aim provided information whether genes involved in proliferation are regulated by BMI1 gene. These data were validated by western blot analysis. We analyzed the expression level of Cyclin D1, p16 and Bcl-2 protein in CaP cells. Next we investigated whether the overexpression generates the data contrary to what was observed in BMI1 silenced cells. For this purpose BMI1 was overexpressed in LNCaP, PC-3 and DU145 cells by transfecting pbabe-BMI1 plasmid. Cell lysates prepared from these cells were analyzed for Cyclin D1, Bcl-2 and p16 proteins by employing western blot analysis. To understand the mechanism through which BMI1 regulates Cyclin D1, we carried out experiments on critical pathways which are already know to be associated with Cyclin D1 expression. This includes Wnt/ β -catenin signaling pathway. We asked whether BMI1 has any association with Wnt/ β -catenin signaling (which is itself reported to control Cyclin D1). Interestingly, we found that BMI1 overexpression causes an increase in the transcriptional activation of *TCF*-responsive element (a bio-marker of Wnt signaling) in CaP cells. Since Bcl-2 was observed to be modulated by BMI1, we investigated if BMI1 has any association with sonic hedgehog (SHH) pathway that is very well know to regulate Bcl-2. For this purpose we determined the expression level of Bcl-2 in BMI1-overexpressing and BMI1-silenced CaP cells in presence of Cyclopamine, a SHH pathway inhibitor. We also tested if re-introduction of BMI1 would restore the Bcl-2 levels in CaP cells pre-treated with

cyclopamine (SHH inhibitor). Further, we investigated an association of tcf and Bcl-2 in CaP cells. We investigated the mechanism through which BMI1 drives the Tcf/Bcl-2 signaling in CaP cells.

Experimental Design for Specific Aim #2.

Animal studies showed a significant lower tumor growth in PC-3-empty vector and PC-3-BMI1-suppressing cell-originated tumors than PC-3-BMI1-overexpressing cell originated tumors in athymic mice. We showed that knocked down of BMI1 sensitized the chemoresistant prostatic tumors for the Docetaxel therapy.

Material and Methods:

Cell Lines and plasmids: Primary prostate epithelial cell (PrEC) was procured from Cambrex BioScience (Walkersville, MD). Normal prostate cell line (RWPE1), CaP cell lines (LNCaP, PC3 and Du145), and colon cancer cell line HT29 were obtained from ATCC (Manassas, VA). pGeneClip-BMI1-shRNA plasmid was procured from SA-Biosciences (Fredrick, MD). pTK-TCF-Luc (TopFlash & FopFlash) was procured from Millipore (Temecula, CA).

Tumor tissues: Frozen prostatic tissues (surgically obtained from CaP patients) and tissues in paraffin blocks, and tissue microarrays were procured from NCI-sponsored Cooperative Human Tissue Network (CHTN, Mid-West Division, University of Ohio, Columbus).

Chemicals and reagents: Docetaxel, casodex, cyclopamine and cisplatin were purchased from LKT Laboratories (St. Paul, MN). Puromycin, G418 and BrdU labeling reagent were purchased from Invitrogen (Carlsbad, CA). The anti-BMI1 antibody, ChIP-grade anti-TCF1 and anti-TCF4 antibody was obtained from Millipore. Anti-BCL2 and anti-BrdU antibodies were purchased from Cell Signaling (Danvers, MA).

Cell growth assay. Cell growth was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-5-diphenyl tetrazoliumbromide; Sigma, Saint Louise, MO, USA) assay as described earlier.²⁷⁻²⁸ Briefly, transfected cells were grown in complete medium. Each condition was repeated in 10 wells. After incubation for specified time at 37 °C in a humidified incubator, MTT (5 mg/ml in phosphate buffered saline, PBS) was added to each well. After 2 h of incubation with MTT, the plates were centrifuged at 500 g for 5 min. After careful removal of the solution, 0.1 ml of DMSO was added to each well and plates were shaken. The absorbance was recorded on a microplate reader at the wavelength of 540 nm. The cell growth was assessed as percent cell growth where vehicle-treated cells were taken as 100% viable.

3[H]-thymidine incorporation assay: 3[H]-thymidine incorporation assay was performed as described earlier.²⁷ Briefly, Cells grown in 24-well plates in the presence of 3[H]thymidine (0.5 µCi/ml). Cells were then washed twice with cold PBS and then were incubated with trichloroacetic acid solution on ice for 30 min. Next, acid-insoluble fraction was dissolved in 1 ml of NaOH (1 M).

Incorporated 3[H]thymidine were quantified using a scintillation counter.

Colony formation assay: A total of 0.5% agar was prepared in appropriate culture media containing 20% fetal calf serum (bottom layer). Cells (1×10^5 cell/ 100 mm plate) in 20% fetal calf serum and 0.7% agarose (top layer) were plated and incubated at 37°C. The medium was removed and replaced with fresh medium in every 2 days. After 14 days of incubation, the cells were stained with 0.05% crystal violet/methanol for 2 h and colonies were counted in two colony grids using a microscope.

Immunohistochemistry. Briefly, paraffin embedded sections (to be evaluated for BMI1, BrdU and BCL2) were treated with Retrieval A solution (pH 6) for antigen retrieval (BD Biosciences, San Diego, CA). Sections were incubated with primary antibody for overnight at 4°C. Slides were then washed and incubated for 2 h at room temperature with appropriate HRP-conjugated secondary antibody. Finally, slides were developed in 3, 3'-diaminobenzidine (DAB kit, Invitrogen) and counter stained with hematoxylin. The stained slides were dehydrated and mounted in permount solution.

Western blot Analysis. Briefly, cell lysates were prepared by incubation of cells for 30 min in ice-cold lysis buffer [(0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mole/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4 , 0.5% NP-40, 1% Triton X-100, 1 mol/L phenyl methylsulfonyl fluoride (pH 7.4)] with protease inhibitor cocktail (Roche, Indianapolis, IN). The lysate was collected; insoluble materials were removed by centrifugation at 4°C for 15 minutes at 15,000g, and stored at -80 °C. BCA protein estimation kit was used to estimate the protein concentration in the lysates (Pierce, Rockford, IL), as per the vendor's protocol. Next, 40 µg protein was resolved in 10% SDS-PAGE gels, transferred onto PVDF membranes (Millipore) and incubated in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20mmol/L TBS, pH 7.6) for 2 h. The blots were incubated with appropriate primary antibody, washed and incubated with HRP-conjugated secondary antibody (Sigma). The blots were detected with chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ). Equal loading of protein was confirmed by stripping the blots and re-probing with α -actin (Sigma).

Luciferase reporter activity. In these studies, cells were co-transfected with the pTK-TCF, (200 ng/well) and pGeneClip-BMI1-shRNA or pbabe-BMI1. Renilla luciferase (pRL-TK) was used as an internal control and reporter activity was measured as described earlier.²⁷⁻²⁸ For controls, the similar amount of empty vectors (pGL3, pbabe and pGeneClip) was transfected in cells.

Quantification of apoptosis. Apoptosis was evaluated using the Annexin V-FITC Apoptosis detection kit from MBL International Corporation (Watertown, MA). Briefly, docetaxel resistant and BMI1-silenced docetaxel resistant cells were harvested with 0.025% trypsin + 5 mM EDTA in PBS (containing 2.5% FBS). Then the cells were washed with PBS and incubated for 5 min at room temperature with Annexin V-FITC plus propidium iodide (PI) as per vendor's protocol. Cells were

analyzed on a Becton Dickinson FACS Calibur flow cytometer (BD Biosciences), placing the FITC signal in FL1 and the PI signal in FL2. Intact cells were gated in the FSC/SSC plot to exclude small debris. Cells in the lower right quadrant of the FL1/FL2 dot plot (labeled with Annexin V-FITC only) are considered to be in early apoptosis, and cells in the upper right quadrant (labeled with Annexin V-FITC and PI) are in late apoptosis/necrosis.

Generation of stable cell lines: PC3 cells were transfected with either pbabe-BMI1 (BMI1-overexpressing) or pGeneClip-BMI1-shRNA (BMI1-silenced) using Lipofectamine and selected in presence of puromycin (1 μ g/ml) and G418 (400 μ g/ml), respectively.

(i). Stable BMI1-overexpressing CaP Cells: For this purpose PC-3 cells were stably transfected with pbabe-BMI1. The transfections were performed by Lipofectamine method. BMI1 overexpressing PC-3 clones were selected in presence of puromycin. The selection of BMI1-overexpressing PC-3 cells under puromycin continued for 4 weeks. Cells were tested for BMI1 overexpression. Among 24 clones generated, we selected 3 clones those exhibited the highest degree of BMI1 expression level.

(ii). Stable BMI1-silenced CaP Cells: For this purpose PC-3 cells were stably transfected with vector-based shRNA plasmid, pGeneCLIP-BMI1-shRNA. The shRNA plasmids are designed using an experimentally validated algorithm. These constructs specifically knock down the expression of specific genes by RNA interference and allow for enrichment or selection of transfected cells. Each vector expresses a short hairpin RNA, or shRNA, under control of the U1 promoter and neomycin gene. Neomycin resistance permits selection of stably transfected cells. The ability to select or track and enrich shRNA-expressing cells brings RNA interference to cell lines with lower transfection efficiencies. Unlike siRNA, plasmid-based shRNA also provide a renewable source of RNA interference reagent. The transfections were performed by Lipofectamine method. PC-3 cells were selected in presence of neomycin analogue G418 (300 μ g/ml). The selection of BMI1-overexpressing PC-3 cells under G418 continued for 4 weeks. Cells were tested for BMI1 expression. Among 20 clones generated, we selected 3 clones those exhibited the least or no BMI1 expression level.

Senescence-associated β -Galactosidase analysis: Senescence-associated β -galactosidase (SA- β -gal) cytochemistry was performed by using X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) substrate kit (Cell Signaling) as per vendor's protocol.

Chemosensitivity assay: Transfected cells (BMI1-overexpressing and BMI1-suppressed) at 12 h post-transfection time period were treated with casodex (10 μ M), docetaxel (10 nM) and cisplatin (10 μ M) for additional 24 h. Growth of cells was determined by measuring rate of proliferation and viability by employing using ³[H]thymidine incorporation and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide] assay as described (13-15)

Generation of chemoresistant CaP cells: One million cells were seeded in 100 mm tissue culture dish with advanced-RPMI (Invitrogen) media containing 5% FBS and 1% penicillin /streptomycin for 24 h. At 24 h, the cells were exposed to 10 nM docetaxel for 48 h. The selection of concentration was based on the IC₅₀ value. The drug containing medium was replaced after two days and the surviving (adherent) cells were cultured in a fresh drug-free complete medium. The cycle was repeated total 10 times. Following each treatment, cells were allowed to fully recover before next treatment. Next, the adherent cells were collected and exposed to higher doses of docetaxel (15, 20, to 25 nM) for 6 weeks (two weeks for each concentration). Finally, the surviving cells were maintained in RPMI/5% FBS containing 10 nM docetaxel. Untreated PC3 cells aged alongside the treated cells (to avoid aging effect) were considered as control.

CaP-specific membrane hybridization and quantitative RT-PCR array: The membrane (printed with probes for CaP-specific genes) was hybridized with cRNA (synthesized from RNA of cells) and detected by chemiluminescence as per vendor's protocol (SA-Biosciences Super Array, Frederick, MD). Further, RNA from cells was also used for performing qRT-PCR array of CaP specific genes. The data analysis was performed by using array analyzer software (SA-Biosciences Super Array, Frederick, MD).

Quantitative chromatin immunoprecipitation (ChIP) assay: ChIP analysis for TCF1 and TCF4 occupancy on promoter region of BCL2 gene was performed as described (14). samples were cross-linked with 1% formaldehyde. Anti-AR antibodies were used with protein A-Sepharose (Sigma) to adsorb immune-specific complexes. Preimmune serum was used as a control. Purified DNA was analyzed by real-time PCR (ABI Prism 7500) using appropriate primers. Product was measured by SYBR green fluorescence and the amount of product was calculated by determining relative expression to a standard curve generated from a titration of input chromatin. Following primers were used to amplify segments that overlap with the appropriate regions: BCL2 (-3.91Kb), Forward, 5'CTGTGGGAGCAAAGGAAGAC3'; Reverse, 5'AGAAGGAAACGGATCCCCTA3'; BCL2 (P2-promoter, TATA site), Forward, 5'CAAGTGTTCCGCG`TGATTG3'; Reverse 5'CCCGGTATCGTACCCTGTT3'; BCL2 (-0.8Kb), Forward, 5'GTCCAAGAATGCAAAGCACAA3'; Reverse-5'CCCCCAGAGAAAGAAG AGGA3'; SP5 (promoter) - Forward 5'-GGGTCTCCAGGCGGC AAG-3'; Reverse, 5'-AGCGAAAGCAAATCC TTTGAA-3'.

Tumor studies in animals: Athymic (nu/nu) male nude mice (6 weeks old; HarlanTek, Madsion, WI), were implanted with stable transfected PC3 cells (1 x 10⁶) in 50 µl RPMI + 50 µl Matrigel (BD Biosciences, Bedford, MA) subcutaneously into the right flanks of each mouse. At 7th day post-implantation, the study was divided into two protocols.

Overexpression protocol. Group-1 (n=10) of mice implanted with empty-vector transfected stable cells and treated with vehicle alone served as control. Group-II (n=10) included mice implanted with vector transfected cells and treated with an intraperitoneal (*i.p.*) administration of docetaxel (10 mg/kg in 100 μ l of saline; thrice/week). Group-III (n=10) included mice implanted with BMI1-overexpressing cells and received *i.p.* administration of 100 μ l of saline. Group-IV (n=10) included mice bearing BMI1-overexpressing tumors and received docetaxel (thrice/week).

Silencing protocol. Group-1 (n=10) of mice implanted with empty-vector (pGenCLIP) transfected stable cells and treated with vehicle served as control. Group-II (n=10) included mice implanted with vector transfected cells and treated with docetaxel (10 mg/kg). Group-III (n =10) included mice implanted with BMI1-silenced tumor cells and received *i.p.* administration of saline. Group-IV (n = 10) included mice implanted with BMI1-silenced tumor cells and received docetaxel treatment.

Tumor measurement: Body weights were recorded seven days/week throughout the study. Tumor growth was recorded as described (13-15). Tumors from three animals from control and treated groups were excised at the 35th day post administration when 100% of control animals reached the preset end point of tumor volume of 1,000 mm³. Rest of the animals in other groups remained under protocol for a maximum time of 10 weeks. Before 2 h of sacrifice, each animal received an *i.p.* administration of BrdU labeling reagent (10 ml/ kg) to label proliferating cells within tumors (13). All procedures conducted were in accordance with the IACUC guidelines.

Statistical analyses: Student's *t* test for independent analysis was applied to evaluate differences between the treated and untreated cells with respect to the expression of various proteins. A Kaplan-Meier survival analysis with the corresponding Log-Rank and Linear Regression analysis was used to measure the rate of mean tumor volume growth as a function of time. A p-value of < 0.05 was considered to be statistically significant.

Key Research Accomplishments:

Under the time period (10/01/2011 to 09/30/2012), we accomplished several goals as proposed under tasks 2 and 3 (as described in the Statement of Work-SOW). These are described as following:

Task 2 (of SOW): To evaluate the effect of BMI1 overexpression and silencing on SHH and Wnt/ β -catenin signaling pathways and to define the underlying mechanism of SHH-BMI1- β -catenin interaction at transcriptional and translational level in human CaP cells. :

Status: Completed; Data presented in this report.

Task 3 (OF SOW): Sub-task: (3A) Studies in athymic nude mouse xenograft model will be conducted (a) to analyze the consequences of BMI1 overexpression and silencing on tumorigenicity of human CaP cells under *in vivo* situation and (b) to evaluate the effect of BMI1 siRNA in combination with inhibitors of SHH and β -catenin signaling on the growth of tumors.

Status : 50% of proposed studies completed (presented in this report), and rest of 50% experiments are underway.

Results :

BMI1 protein levels in prostatic tissues increases with progressive stages of disease in human CaP patients: As an attempt towards identifying the expression status of BMI1 protein in human prostatic tissues, we measured its expression levels by performing immunoblot analysis of snap-frozen prostatic tissues from normal, dysplasia and CaP patients (stage II-IV) procured from NCI-sponsored Cooperative Human Tissue Network (CHTN, Mid-West Division, University of Ohio, Columbus). BMI1 protein was observed to be progressively increased from normal prostate to prostatic dysplasia to malignant prostate in humans (Fig. 1A).

We next determined the expression of BMI1 protein in 72 pair-matched specimens of normal and CaP representing all tumor stages by employing immunohistochemical analysis. The intensity of immunoperoxidase staining for BMI1 was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Immunostains showed granular cytoplasmic staining in both non-neoplastic and neoplastic epithelium. In general, the staining was stronger in neoplastic epithelial cells than in non-neoplastic epithelial cells (Fig. 1Bi). We next compared the staining pattern of BMI1 protein of stages II-IV CaP specimens. As shown in box-plot, a progressive increase of BMI1 was observed as the disease progressed from low-grade to high grade tumor in humans CaP patients (Fig. 1Bii). The box plots of the data for BMI1 protein expression in epithelial cells exhibited a wide inter-specimen variation in cancer specimens, compared with normal tissues and revealed a significant difference in the level of protein between normal and CaP tissues ($p < 0.05$, Fig. 1Bii). The average score for the staining intensity of BMI1 in normal tissues was 1.3 ± 0.07 ($n=72$), and was significantly lower than high-grade stage II (1.9 ± 0.09 ; $n=36$), stage III (2.4 ± 0.08 ; $n=28$) and stage IV (2.92 ± 0.08 ; $n=6$) cancer specimens (Fig. 1Bii; $p < 0.05$). Taken together, these data show that expression of BMI1 increases with increasing stage of CaP.

BMI1 expression is independent of androgen: We next investigated if the observed increase in BMI1 levels during the progression of CaP disease in humans has a correlation with presence or absence of androgen. Androgen analogue (R1881; 1 nM) treated LNCaP, LAPC4, 22Rv1 and C4-2b cells did not show any significant change in the levels of BMI1 protein (Fig. 1C) suggesting that BMI1

expression is independent of influence of androgen.

BMI1 regulates the proliferation of prostatic tumor cells: We investigated whether BMI1 regulates the growth of prostatic tumor cells. We employed a two-way approach where BMI1 was (i) either suppressed by transfecting a vector-based shRNA (pGenCLIP-BMI1-shRNA) or (ii) overexpressed by a ectopic transfection with BMI1 expressing plasmid (pBabe-BMI1) in prostatic tumor cells (LNCaP, Du145 and PC3) (Suppl. Fig. 1). The effect of differential expression of BMI1 (overexpression or suppression) on the growth potential of CaP cells was determined by measuring cell viability and the rate of proliferation of cells. LNCaP cells duplicate within 48-72 h while as Du145 and PC3 cells duplication takes 24 h under culture conditions. BMI1-suppressed CaP cells (LNCaP, DU145 and PC3) did grow between 50-65% even after 72 h post-transfection (Fig. 1Di). However, BMI1-overexpressing CaP cells achieved 65-90% confluence only at 36h post-transfection suggesting the significance of BMI1 in the growth of tumor prostatic cells (Fig. 1Dii).

We next asked if the pro-growth role of BMI1 is due to its effect on the proliferative potential of tumor cells. For this purpose, we employed 3[H]thymidine uptake assay that measures the rate of uptake of thymidine by dividing/proliferating cells. As evident from the rate of 3[H]thymidine uptake by proliferating cells, suppression of BMI1 was observed to cause a decrease in rate of proliferation of tumor cells (Fig. 1Ei). Conversely, forced overexpression of BMI1 caused a significant increase in the rate of proliferation ($p < 0.05$, Fig. 1Eii). Since, we used vector-based shRNA that provides an advantage to investigate the effect of BMI1 suppression over a long period in cells; we also assessed proliferative potential of tumor cells by employing soft-agar colony formation assay. BMI1-suppressed tumor cells exhibited significantly reduced number of colonies formed ($p < 0.05$, Fig. 1Fi). On the contrary, forced overexpression of BMI1 induced clonogenic potential of tumor cells as was evident from the number of colonies formed by these cells (Fig. 1Fii). These data suggested that BMI1 confers proliferative attributes to the CaP cells.

BMI1 increases the replicative life of normal prostate epithelial cells (PrEC): To further confirm that BMI1 indeed drives the proliferation of epithelial cells, we selected normal primary epithelial cells (PrEC). It is well known that normal prostate cells enter into senescence and do not grow or replicate after 4-5 passages under culture conditions. It is noteworthy that forced expression of BMI1 in normal cells (transfected with pbabe-BMI1) abolished senescence and caused an increase in the replicative cycles (Fig. 2A). This was evident from the number of passages (8 passages) that we were able to subculture BMI1-overexpressing normal cells. Due to transient nature of transfection, the effect of overexpression lasted up to 7th passage, and cells entered into senescence at 8th passage. To further confirm if the increase in replicative potential (conferred by BMI1 overexpression) was due to abolishment of senescence in normal cells, we measured senescence-associated β -galactosidase

(SA- β -gal) activity, a general marker of senescence, at various passage/replication levels in PrEC cells. By employing a cytochemical reaction that is indicative SA- β -gal activity, normal cells exhibited positive staining for SA- β -gal at the 4th passage/subculture. Notably, BMI1 overexpressing normal cells did not show positive staining for SA- β -gal at the 4th passage. BMI1-overexpressing normal cells showed for SA- β -gal activity at the 7th passage (Fig. 2B). These data further strengthen our hypothesis that BMI1 has the potential to drive both normal and neoplastic cells towards proliferation though its activity is increased in neoplastic cells.

Molecular mechanism of action of BMI1 in tumor cells: We investigated the mechanism through which BMI1 controls the proliferation of CaP cells. For this purpose we performed focused membrane and quantitative-PCR based array analysis of well-characterized genes known to be involved in the proliferation of CaP cells. Since LNCaP cells exhibit the expression of majority of the human genes, we performed the primary analysis in these cells. A cut-out point of 2-fold was selected for analysis. To observe if the effect of BMI1 suppression (as observed in LNCaP cells) was similar to all CaP cells, the data were further validated in DU145 and PC-3 cells. BMI1-suppressed LNCaP cells exhibited a significant change in the expression level of several proliferation-associated genes (Fig. 1C & Suppl. Table-1). The prominent genes downregulated by suppression of BMI1 in cells were Cyclin D1, BCL2, IL and NF κ B (Fig. 1C & Suppl. Table-1). An increased expression of p16, p15 and TIMP3 in BMI1-suppressed tumor cells was observed (Fig. 1C & Suppl. Table-1 & Figure 2A). To validate whether changes induced by BMI1 suppression on gene transcripts translated to protein level, we performed immunoblot analysis for selected gene products in LNCaP, DU145 and PC3 cells. BMI1-silenced tumor cells exhibited decreased levels of Cyclin D1 and BCL2, and increased p16 (Fig 2D). These data were further validated in BMI1 overexpressing cells which were observed to display increased BCL2 and CyclinD1 in cells (Fig. 2E). The data suggested a possible association between the BMI1, BCL2 and Cyclin D1 in CaP cells.

BMI1 in survival, growth and chemoresistance of tumor cells: Since BMI1 was observed to regulate the expression of proliferation-associated genes, we sought to determine if this phenomenon is responsible for chemoresistance of CaP cells. By employing 3[H]thymidine uptake and cell viability assays, we next determined the growth potential of BMI1-overexpressing and –suppressing CaP cells treated with clinically used chemotherapeutic drugs (bicalutamide or casodex, docetaxel and cisplatin). The working concentrations of the drugs were determined by the time course and dose titration for the chemotherapeutic agents (Suppl. Fig. 2C-D). We avoided casodex treatment in androgen-independent PC3 cells. The 3[H]thymidine incorporation analysis of cells showed that BMI1-overexpressing tumor cells significantly were non-responsive to chemotherapeutic agents suggesting that BMI1-rich tumor cells harbor the potential of escaping the chemotherapy (Fig. 3B &

D). On the contrary, BMI1-suppressed prostate tumor cells were significantly responsive to chemotherapeutic treatments and exhibited reduced rate of proliferation suggesting that abolishing BMI1 render tumor cells sensitive to the chemotherapy ($p < 0.05$, Fig. 3A & C). The observed differences between the BMI1-overexpressing and BMI1-suppressed tumor cells in responsiveness towards chemotherapy were also reflected in cell viability (Suppl. Figs. 3 & 4). Cell viability data was concomitant to the 3[H]thymidine incorporation data (Suppl. Figs. 3 & 4). These data established the significance of BMI1 protein in cell growth and suggest that presence of BMI1 in tumor cells play a critical role in deciding the therapeutic outcome.

BMI1 is critical for regrowth of tumors post-chemotherapy: We conducted a proof of principle study where adherent PC3 cells which survived docetaxel treatment were repopulated and allowed to regrow. These regrown CaP cells were termed as chemoresistant as these did not responded to subsequent docetaxel treatments and exhibited increased expression levels of BMI1, BCL2 and Cyclin D1, and rate of proliferation (Fig. 2Ei-ii). We next asked if targeting BMI1 render the BMI1-rich chemoresistant tumor cells amenable to therapy. Chemoresistant CaP cells were subjected to BMI1-suppression by shRNA. 3[H]thymidine uptake and FACS analysis for Annexin V/PI staining of cells showed that targeting BMI1 significantly inhibited the proliferation of chemoresistant CaP cells, increased the apoptosis and caused a remarkable decrease in the levels of BCL2/Cyclin D1 (Fig. 2F-H) suggesting a possible association between BMI1 and the survival of tumor cells post-chemotherapy.

Molecular mechanism through which BMI1 regulate BCL2 in tumor cells: Cyclin D1 and BCL2 are both down-stream targets of Wnt and Sonic hedgehog (Shh) signaling (16-17). Therefore, we determined if BMI1 has (i) any direct or indirect association with Wnt and Shh-signaling in proliferating tumor cells, and (ii) regulates BCL2 under Wnt or Shh guidance. We first determined the status of Wnt-signaling in BMI1-suppressed and BMI1-overexpressing CaP cells. By utilizing transcriptional reporter activity of TCF element (a biomarker for Wnt activation), we observed that BMI1-overexpressed cells harbor increased TCF-transcriptional activity concomitant with the increased BCL2 and Cyclin D1 expression (Fig. 4A). Notably, BMI1-silenced tumor cells exhibited decreased TCF-transcriptional activation (Fig. 4B). Since our data showing a regulatory role of BMI1 on TCF-transcriptional activity in tumor cells is novel, we asked if this phenomenon is only limited to CaP cells. To validate our observations, we selected colon cancer cell line HT29 which is known to exhibit increased Wnt signaling and TCF-transcriptional activity (18). We first determined BMI1 levels in HT29 cells and then generated BMI1-silenced and BMI1-overexpressed HT29 cells by transfecting plasmids for BMI1-silencing and -overexpression, respectively. Significant modulations of TCF-transcriptional activity concomitant with BCL2 and Cyclin D1 were observed in HT29 cells after BMI1-

silencing or -overexpression (Fig. 4C-E). These data established the role of BMI1 on TCF-transcriptional activity in other tumor cells.

Since BCL2 is also a downstream target of Shh, we next determined if BMI1 (i) partially regulated BCL2, or (ii) regulation of BCL2 is solely under the control of Shh as reported (18). For this reason, CaP cells were treated with cyclopamine (5 μ M), an inhibitor of Shh signaling. As expected control (vehicle treated) CaP cells exhibited a reduction in BCL2 expression after cyclopamine treatment, however BMI1-overexpressing tumor cells did not show an apparent decrease in BCL2 (Fig. 4Fi). Furthermore, when BMI1 was suppressed in BMI1-rich tumor cells, they responded well to cyclopamine and exhibited reduced BCL2 levels (Fig. 4Fii). To validate if BMI1 was involved in regulation of BCL2 independent of Shh, we next reintroduced BMI1 in (a) cyclopamine treated, and (b) BMI1-deficient CaP cells. Reintroduction of BMI1 in cyclopamine treated and BMI1-suppressed cells caused a gain in BCL2 promoter activity and expression level suggesting that BCL2 expression is not solely under the control of Shh-signaling but is in part regulated by BMI1 (Fig. 4G).

Since both TCF and BCL2 were observed to be under the guidance of BMI1, we next investigated whether there is a direct association of TCF and BCL2 gene in tumor cells. We measured BCL2 activity under condition when TCF was suppressed in cells. For this purpose, LNCaP, PC3 and HT29 cells were co-transfected with TCF-shRNA and BCL2-luc reporter plasmid and were analyzed for BCL2-promoter activity after 36 h. TCF-suppressed CaP and HT29 cells exhibited decreased BCL2-promoter activity (Fig. 4H). This report suggests that BCL2 acts as a downstream target of TCF-mediated signaling pathway.

TCF4 transcriptional factor binds to promoter region of BCL2 gene in tumor cells: To further identify the underlying mechanism, we investigated whether BCL2 gene has possible TCF binding sites on its promoter. By employing TESS analysis, we observed that BCL2-promoter region exhibits multiple sites where TCF possess the affinity to bind. We next sought the validation of TESS data (which is a mathematical data) by biochemical analysis. We tested TCF1 and TCF4 occupancy on multiple sites on the promoter region of BCL2 gene by employing ChIP assay in CaP and HT29 cells. The TCF unresponsive SP5 promoter was used as a negative control (data not shown). TCF1 was observed to have insignificant occupancy on the examined sites of BCL2-promoter in both CaP and colon cells (data not shown). Among the three region analyzed, TCF4 was observed to occupy only TATA region of BCL2-promoter (P2 promoter). We found very little or no occupancy by TCF4 on -3.41Kb and -8.41kb of BCL2 promoter (data not shown). Notably, BMI1-overexpression was observed to increase the TCF4 occupancy on the TATA region of BCL2-promoter in the PC3 and HT29 cells (Fig. 5A-B). An opposite result was observed with that of BMI1-suppressed CaP and HT29 cells (Fig. 5A-B).

TCF4 binds to promoter region of BCL2 gene in human prostatic tissues: We next investigated if the *in vitro* observation of TCF4 occupancy on BCL2-promoter binding has translational relevance. From the outcome of immunoblot analysis data (Fig. 5C), we selected human normal and malignant prostatic tissues (which exhibited increased BMI1) and determined TCF4-occupancy on BCL2-promoter. As compared to the normal prostatic tissues, TCF4 exhibited increased occupancy at TATA region of *BCL2* in human prostate tumor tissues (Fig.5D).

BMI1 confers chemoresistance to human prostatic tumors in xenograft mouse models: Since BMI1 was observed to be involved in the proliferation and chemoresistance of CaP cells to various antitumor agents under *in vitro* conditions, we next determined whether these observations could be translated under *in vivo*. For this purpose, we measured the differential growth rate of tumors-derived from BMI1-overexpressing and –suppressing PC3 stable cells under two protocols.

BMI1-overexpression protocol: At one week post-implantation, the average volume of control and BMI1-overexpressing tumors in mice increased as a function of time (Fig. 5E). BMI1-overexpressing tumors were observed to grow at faster rate and larger in size than control tumors (Fig. 5E). Mice implanted with control-tumors reached a preset end-point tumor volume of 1000 mm³ at 49th day of post-implantation (Fig. 5E). However, mice implanted with BMI1-overexpressing tumors reached the preset end-point tumor volume at 35th day of post-implantation (Fig. 5E). At 49th and 35th days, mice implanted with control and BMI1-overexpressing tumors exhibited average tumor volumes of 1076 and 1023mm³, respectively (Fig. 5E).

Docetaxel treatment decreased the growth of tumors in control mice (Group-II). Interestingly, docetaxel treatment failed (for 10-weeks) to produce an effect on the growth of tumors expressing high BMI1 protein. At 49th day, the average tumor volume in control mice (Group-II) treated with docetaxel was 850 mm³ (Fig. 5E). However, BMI1-overexpressing tumors though treated with docetaxel reached an average tumor volume of 997 mm³ as early as 35th day (Fig. 5E). Next, we evaluated whether treatment of docetaxel to animals caused a delay in the growth of tumors harboring increased BMI1 levels. As evident from the number of mice reaching the preset end-point tumor volume (1000 mm³), the observed differences between the control and BMI1-overexpressed group of animals for the outcome of docetaxel therapy was statistically significant ($p < 0.05$; Fig. 5F).

BMI1-suppression protocol: BMI1-suppressed cell-derived tumors were observed to grow at slower rate than control tumors (Fig. 5G). This was evident from the significant difference in the rate of growth and tumor volumes between control and BMI1-suppressed group of animals (Fig. 5G). Mice implanted with control tumors reached a preset end-point tumor volume of 1000 mm³ at 49th day of post-implantation (Fig. 5G). It is noteworthy that the average volume of tumors in mice bearing BMI1-silenced tumors did not reach the end-point even at 70th day post-implantation (Fig. 5G). At 49th day,

control group of animals treated with docetaxel exhibited an average tumor volume of 850 mm³. However at this point, BMI1-silenced group of animals treated with docetaxel exhibited an average tumor volume of 230 mm³ suggesting that BMI1-silencing sensitizes tumor cells to docetaxel therapy (Fig. 5G). Next, we evaluated whether docetaxel caused a delay in the growth of BMI1-suppressed tumors. The observed differences between control and BMI-silenced group of animals were statistically significant ($p < 0.05$, Fig. 5H).

BMI1 controls proliferation and BCL2 expression of tumor cells in vivo: We determined significance of BMI1 in the proliferation of cells within tumors exposed to docetaxel therapy. Proliferating tumor cells are known to uptake BrdU (thymidine analog) and its detection in tumor cells by immunostaining of tumors provides an indirect measure of *in vivo* proliferation. Notably, BrdU staining of tumor sections (harvested at 35th day) showed that docetaxel treatment decreased the number of proliferating cells in BMI1-silenced tumors and failed to inhibit proliferation of BMI1-overexpressing tumor cells (Fig. 6A). BMI1-silenced tumors exhibited decreased BCL2 levels than BMI1-overexpressing tumors (Fig. 6B). These data show that BMI1 confers chemoresistance to prostatic tumors, and abolishing BMI1 sensitizes chemoresistant tumors to chemotherapy, therefore establishes its significance as a therapeutic target.

Reportable Outcome: Based on our results, three major observations were found to be reportable. Two of these observations were submitted for their publication in scientific journals. A part of our observations has now been accepted for its publication by scientific journal “PLOS-One”.

On the basis of our studies, following major observations were made:

1. BMI1 protein levels progressively increase with the advancement of CaP disease in humans. In addition, BMI1 protein is increased in disease which is resistant to therapy. The significance of this outcome is that tissue biopsies in future could be analyze for BMI1 protein to assess if the disease would be aggressive and treatable or not. BMI1 would act as a future biomarker. This would save time of clinicians to manage the CaP disease in men. This is an important reportable outcome.
2. The significance of this study is that chemoresistant prostatic tumors could be treated now by targeting BMI1. This study open up the opportunities to develop new drugs and therapies those specifically could target BMI, thus could be used to overcome therapy resistance of hard-to treat tumors. This is an important outcome of this study.

Conclusion

Recent studies showed that dysregulation of BMI1 alters cell proliferation, senescence and self-renewal of several human cancers (6,10). BMI1 is reported to play a crucial role during epithelial

mesenchymal transition during tumor development (6,20). However, the role of BMI1 in human CaP progression and chemoresistance is not well studied (21). It is speculated that inability of tumor cells to undergo apoptosis in response to chemotherapy results in a selective advantage for such tumor cells to become more aggressive compared to chemoresponsive cells during progression of CaP (6). Several studies demonstrate that BMI1 rescues tumor cells from apoptosis and could be a critical factor involved in the emergence of chemoresistance, however no concrete mechanism of action is yet known (8-10). Chemoresistant CRPC is hard-to-treat disease and identifying a critical molecule that confers the chemoresistant characteristic to such tumors would be an important advancement in the field of cancer therapy. In the current study, we provide mechanism-based evidence to show that BMI1 plays a critical role in deciding the therapeutic outcome and the fate of tumor cells undergoing chemotherapy. This study is significant because we demonstrated that BMI1 equally confers chemoresistance to hormone-sensitive CaP and CRPC cells. This is further strengthened by the data that BMI1 expression does not get influenced by androgen. Our data is significant because it explains the possibility of BMI1 as a part of the mechanism that drives indolent disease to aggressive phenotype which is often androgen-independent. This observation carries high significance because CRPC tumors in men proliferate under low androgen conditions (5). Based on our data we suggest that targeting BMI1 should be a part of strategy when therapeutic plans are devised to combat chemoresistant type of cancer.

One of the important observations of this study is that BCL2 and Cyclin D1 (found to be regulated by BMI1) have a commonality to also be functional members of Wnt and Shh pathways. Activity of BCL2 and Cyclin D1 are reported to be high in chemoresistant tumors (13,22-24). Keeping in view the critical role of BCL2 in chemoresistance, targeting the protein directly (anti-BCL2 immunotherapy) or blocking the pathways (such as Shh) which regulates its expression, is being suggested as an ideal strategy to overcome chemoresistance of tumor cells (21, 25-26). Shh inhibitor (Cyclopamine) known to downregulate BCL2 in some tumors is currently being investigated as a therapeutic agent for basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, and glioblastoma (27). However the mechanism that causes the resurgence of tumor cells after BCL2-targeted therapy is not known. Our study is significant because we show that BCL2 is not completely lost in tumor cells after chemotherapy and alternate pathways (such as BMI1/TCF4 molecular pathway) regulate BCL2 in chemoresistant tumor cells. This is based on our data showing that (i) BMI1 regulates BCL2 independent of Shh-signaling in chemoresistant tumor cells and (ii) elevated levels of BMI1 and BCL2 in cells those survived chemotherapy. We suggest that this mechanism could be an explanation for the survival of chemoresistant cells post-chemotherapy. Although the previous report showed that BMI1 itself is a target of Shh-signaling, our data show that BMI1 acts independent of Shh

(28). It is possible that chemoresistant cells expressing BMI1 are a highly selected sub-population that remains hard to treat and play an important role in indolence of disease in human CaP patients. BMI1 activity is manifested in the form of repression of target genes such as p16 and the mode of action could be through epigenetic silencing, modulation in the methylation states of genes.⁴ However, in this study we observed that BMI1 upregulates *BCL2* gene. Keeping in view the repressive nature of BMI1, there was a need to understand the mode of action (other than repression) through which BMI1 induces *BCL2* expression and activity. We provided evidence that *BCL2* activation in chemoresistant cells under the guidance of BMI1 is mediated by TCF4 in tumor cells. This was validated in prostate and colon cancer cells *in vitro*; and in human prostatic tissues. We identified the binding regions of TCF4 transcriptional factor on the promoter of *BCL2*. By conducting several ChIP assays, we observed that binding efficiency of TCF4 to the *BCL2*-promoter is dependent on the BMI1 levels. Although the complete information about the regulation of TCF4 by BMI1 is not completely understood, current data suggest that TCF4 indeed is in part under the control of BMI1. The significance of our data is that it (i) identifies BMI1-induced TCF4 as a molecular module that drives Wnt-signaling within chemoresistant tumor cells, and (ii) *BCL2* as a target of BMI1/TCF4 molecular module. Based on our data, we speculate that molecular module could be operational during emergence of chemoresistance in CaP cells and also responsible for the survival and proliferation of chemoresistant tumor cells after chemotherapy.

Docetaxel has been tested under several clinical trials alone and in combination with other agents to treat CaP. Docetaxel therapy was observed to result in a PSA drop of more than 50% in CaP patients, an observation made in several trials such as the SWOG trial (29). However, docetaxel alone, and in combination do not completely abrogate the tumor or bring down PSA levels to the normal in human CaP patients (29). Although effective in CaP patients to an extent, some CaP conditions do not respond to docetaxel therapy and such patients do not exhibit changes in PSA level after therapy (30). In this context, this study is highly significant as we show that targeting BMI1 in chemoresistant CRPC cells sensitizes tumor cells to docetaxel therapy both *in vitro* and *in vivo*. This study identified BMI1 as an ideal molecule to be targeted to overcome the chemoresistance of CaP cells and corroborates to earlier report showing the utility of BMI1 as a target to overcome chemoresistance in ovarian cancer cells (9). Under *in vivo* conditions, the significance between BMI1-positive, BMI1-silenced and BMI1-overexpressed tumor cells vis-à-vis docetaxel therapy was significant. The success of docetaxel therapy against prostatic tumors in a xenograft mouse model was observed to be highly dependent on the level of BMI1. We suggest that preventing the development of chemoresistance in CaP patients will be beneficial for a large group of patients and interventions directed against BMI1 may provide opportunities to enhance the efficacy of

chemotherapy. In this direction we have opened another front by identifying small molecule inhibitors of BMI1. We suggest that these should be explored against chemoresistant tumors. The advanced work with small molecule inhibitors of BMI1 against chemoresistant tumors is underway in our laboratory.

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Legends to Figures

Figure 1. BMI1 Protein levels are increased during the progression of CaP from low to high-grade in human patients and plays an important role in cell proliferation, independent of androgen. (A) Figure represents the level of BMI1 protein in human normal, dysplasia and tumor prostatic tissues as assessed by immunoblot analysis. Equal loading of protein was confirmed by reprobing immunoblot for α -actin. (Bi) Photomicrographs represent immunostaining of BMI1 in CaP specimens and non-neoplastic regions of prostatic specimens of CaP patients. ➔ Arrows indicate staining for BMI1. Magnification X40. (Bii) Box plots for BMI1 protein based on score pertain to immunostaining pattern in normal and CaP specimens. *, $P < 0.05$; black bar in gray box, median values. (C) Figure represents the level of BMI1 protein in androgen (R1881) treated and non-treated CaP cells as assessed by immunoblot analysis. Equal loading of protein was confirmed by reprobing immunoblot for β -actin. (Di-ii) The histogram represents the rate of cell proliferation and % cell growth of CaP cells at 36h post-transfection as measured by MTT assay. (Ei-ii) Histogram showing rate of [3H]thymidine uptake in BMI1-silenced and -overexpressed CaP cells. (Fi-ii) Histogram showing number of colonies formed by BMI1-silenced and -overexpressed CaP cells. Each bar in each histogram (Fig. D-F) represents mean \pm S.E., * indicates $p < 0.05$. All experiments were repeated three times with similar results.

Figure 2. BMI1 play a critical role in the proliferation of normal and tumor cells and regulates the expression of genes associated with the proliferation of prostatic tumor cells. (A)

Photomicrographs represent the effect of BMI1-overexpression on the replication/ proliferation of normal prostate epithelial cells (PrEC). PrEC cells were transfected with pbabe-BMI1 plasmid and equal amount of empty vector (control). Confluent dishes containing BMI1-overexpressing cells and vector-transfected PrEC cells were split or seeded after every 36h. Left Panel: Cell splitting or seeding continued for 5 passages or replication cycles in control PrEC cells and did not duplicate after 5 passages and entered into senescence phase. Right Panel: Cell splitting or seeding continued for 8 passages or replication cycles in pbabe-BMI1 transfected PrEC cells. Inset regions (400X) showing cells with senescent morphology features of live cells such as globular shape. **(B)** Photomicrographs represent the effect of BMI1-overexpression on the senescence-associated β -galactosidase activity in normal prostate epithelial cells (PrEC). PrEC cells were transfected with pbabe-BMI1 plasmid and equal amount of empty vector (control). Confluent dishes containing BMI1-overexpressing cells and vector-transfected PrEC cells were split or seeded after every 36h. Upper Panel: Cell splitting or seeding continued for 4th passages or replication cycles in control PrEC cells and entered into senescence phase. Lower Panels: Cell splitting or seeding continued for 7 passages or replication cycles in pbabe-BMI1 transfected PrEC cells. ➔ Arrows indicate staining for β -galactosidase. **(C)** Scattered Plot for PCR array. The dots indicate the folds-Change of gene regulation. Fold change ($2^{\Delta\Delta Ct}$) is the normalized gene expression ($2^{\Delta Ct}$) in the BMI1 suppressed sample divided the normalized gene expression ($2^{\Delta Ct}$) in the Control Sample. For details please see the Supplementary scatter plot excel data sheet and Clustrogram (Suppl. Fig. 2). **(D & E)** Figure represents the effect of **(A)** BMI1-silenced and **(B)** BMI1-overexpression on the expression level of Cyclin D1, BCL2 and p16 proteins in CaP cells as assessed by immunoblot analysis. For immunoblot data analyses, equal loading was confirmed by reprobing immunoblots for β -actin.

Figure 3. BMI1 confers chemoresistance against chemotherapeutic agents by inhibiting cell death of prostatic tumor cells. (A-D) The histogram represents the rate of proliferation of cells as measured by ^3H -thymidine uptake assay in (A-B) LNCaP and (C-D) PC3 cells harboring varied BMI1 levels treated with different chemotherapeutic agents. Vehicle treated cells were considered as control. **(Ei)** Figure represents the level of BMI1, Cyclin D1 and BCL2 protein in chemoresistant PC3 cells as assessed by immunoblot analysis. **(Eii)** Histogram showing the rate of ^3H -thymidine uptake in chemoresistant CaP cells. **(Fi)** Figure represents the level of BMI1, Cyclin D1 and BCL2 protein in BMI1-silenced chemoresistant PC3 cells as assessed by immunoblot analysis. **(Fii)** Histogram shows the rate of proliferation as assessed by ^3H -thymidine uptake assay in BMI1-silenced chemoresistant CaP cells. Control cells were transfected with empty vector alone. For immunoblot data analyses (Fig. Ei and Fi), equal loading was confirmed by reprobing immunoblots for

□actin. Each bar in the histogram (Fig. A-D, Eii and Fii), represents mean \pm SE of three independent experiments, * represents $P < 0.05$. **(G-H)** Quantitative estimation of apoptosis in BMI1-silencing chemoresistant cells as assessed by flow cytometry. Both docetaxel resistant and BMI1-silenced docetaxel resistant cells were labeled with Annexin-V and PI. Intact cells were gated in the FSC/SSC plot to exclude small debris. Cells in the lower right quadrant of the FL1/FL2 dot plot (labeled with Annexin V-FITC only) are considered to be in early apoptosis, and cells in the upper right quadrant (labeled with Annexin V-FITC and PI) are in late apoptosis/necrosis. The images shown here are representative of three independent experiments with similar results.

Figure 4: BMI1 modulates BCL2 activity in Tumor cells. (A-B) Histogram represents the effect of BMI1-overexpression (A) and –silencing (B) on the transcriptional activation of TCF responsive element in CaP cells as assessed by Luciferase based reporter assay. Relative luciferase activity was calculated with the values from vector alone group. **(C-D)** Histogram represents the effect of BMI1-overexpression (C) and -silencing (D) on the transcriptional activation of TCF responsive element in HT29 cells as assessed by Luciferase based reporter assay. **(E)** Figure represents the effect of BMI1-silencing and -overexpression on the level of BCL2 and Cyclin D1 proteins in HT29 cells as assessed by immunoblot analysis. **(F)** Figures represent the effect of cyclopamine treatment on the level of BCL2 protein in (Fi) BMI1-overexpressing and (Fii) -suppressing LNCaP cells. At 24h post-transfection, cells were treated with cyclopamine in fresh media. Control cells were treated with DMSO (vehicle alone). After 12h incubation of cells with cyclopamine or vehicle alone cells were harvested and immunoblot analysis were performed. **(G)** Histogram represents the effect of cyclopamine treatment on the transcriptional activity of BCL2 promoter in LNCaP cells under BMI1-overexpression and –suppression. **(H)** Histogram represents the effect of TCF-silencing on the transcriptional activity of BCL2 promoter in CaP and HT29 cells as assessed by Luciferase based reporter assay. Relative luciferase activity (G & H) was calculated with the values from vector alone group. For immunoblot data analyses (Fig. E and F), equal loading was confirmed by reprobing immunoblots for β -actin. Each bar in the histogram (Fig. A, B, C, D, G and H), represents mean \pm SE of three independent experiments, * represents $P < 0.05$.

Figure 5. BMI1 regulates BCL2 by occupying its promoter in tumor cells and tissues and confers chemoresistance in a xenograft mouse model. (A-B) Effect of BMI1 expression on TCF4-occupancy on promoter site of BCL2 in PC3 (A) and HT29 (B) cells as assessed by ChIP assay as described in materials and methods. **(C)** Figure represents the level of BMI1 protein in normal and malignant human prostate tissues as assessed by immunoblot analyses. **(D)** Histogram shows the TCF4-occupancy on promoter site of BCL2 in human normal and malignant prostate tissues as assessed by ChIP assay. For immunoblot data analyses, equal loading was confirmed by reprobing

immunoblots for β -actin. Each bar in the histogram, represents mean \pm SE of three independent experiments, * represents $P < 0.05$. **(E & F)** The graphical representation of data showing the effect of docetaxel therapy on the growth of BMI1-overexpressing (BO, A) and –silencing (BS, B) PC3-derived tumor cells implanted in mice. The growth was measured in terms of average volume of tumors as a function of time. Data is represented as mean \pm SE, * indicates $p < 0.05$ from the control group. **(G & H)** The graphical representation of the data showing the number of mice remain with tumor volumes $< 1000 \text{ mm}^3$ after either BMI1-overexpression (C) or BMI1-silencing (D) for indicated weeks. The details are described under Materials and Methods.

Figure 6. Relevance of BMI1 in (A) proliferation and (B) BCL2 expression of tumor cells *in vivo*. Photomicrographs (20x, magnification) showing **(A)** *in vivo* cell proliferation as assessed by BrdU assay and **(B)** BCL2-expression in tumors as assessed by immunostaining. The arrows in the micrographs represent regions exhibiting immunoreactivity. The immunostaining data was confirmed in all specimens from each group (n =10).

Legends to Supplementary figures:

Figure 1. (A) Figure represents the effect of BMI1-silencing and -overexpression on the level of BMI1 protein in CaP cells as assessed by immunoblot analyses. Equal loading was confirmed by reprobing immunoblots for β -actin.

Figure 2. **(A)** Figure represents clustrogram of gene expression as assessed by PCR array. Clustrogram is the representation of three independent experiments. **(B-D)** Time-course and dose titration curves for the chemotherapeutic agents Casodex (B), Cisplatin (C) and Docetaxel (D) as assessed by MTT assay. Vehicle treated cells were considered as control. Data represents mean \pm SE of three independent experiments.

Figure 3: (A-B) The histogram represents the rate of proliferation of cells as measured by MTT assay in BMI1 overexpressing (A) LNCaP and (B) PC3 cells treated with different chemotherapeutic agents. Vehicle treated cells were considered as control. Each bar in the histogram, represents mean \pm SE of three independent experiments, * represents $P < 0.05$.

Figure 4: (A-B) The histogram represents the rate of proliferation of cells as measured by MTT assay in BMI1-silenced (A) LNCaP and (B) PC3 cells treated with different chemotherapeutic agents. Vehicle treated cells were considered as control. Each bar in the histogram, represents mean \pm SE of three independent experiments, * represents $P < 0.05$.

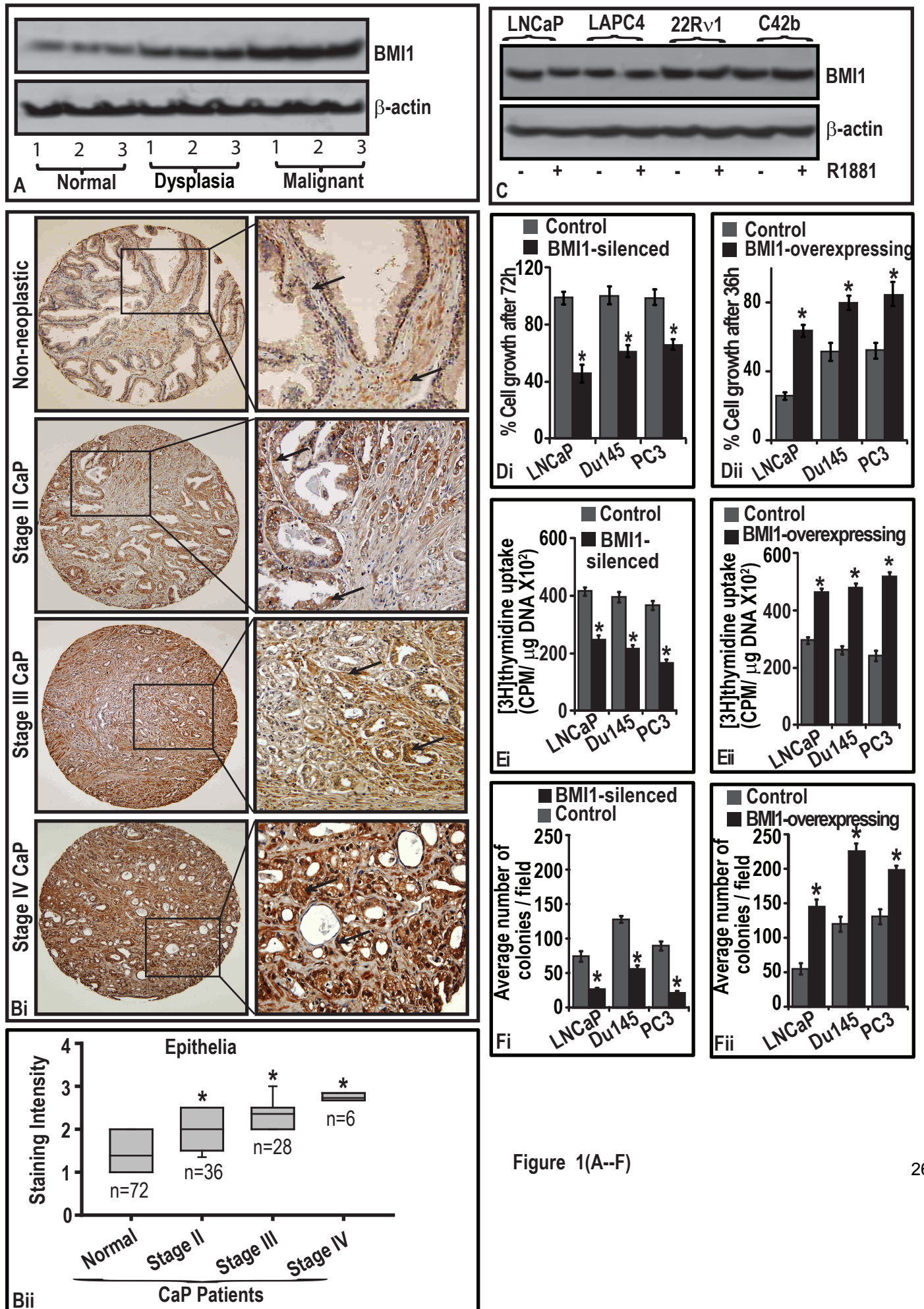


Figure 1(A-F)

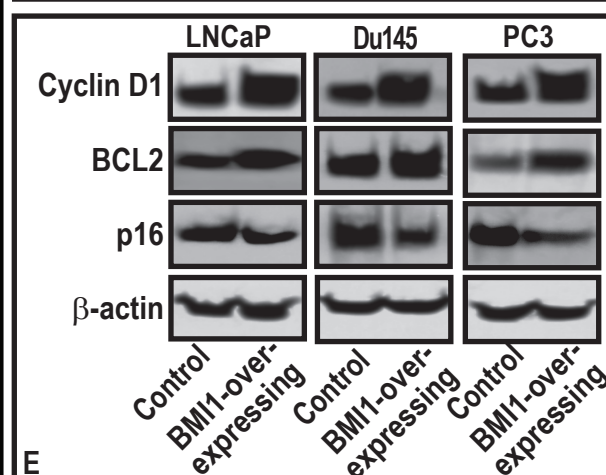
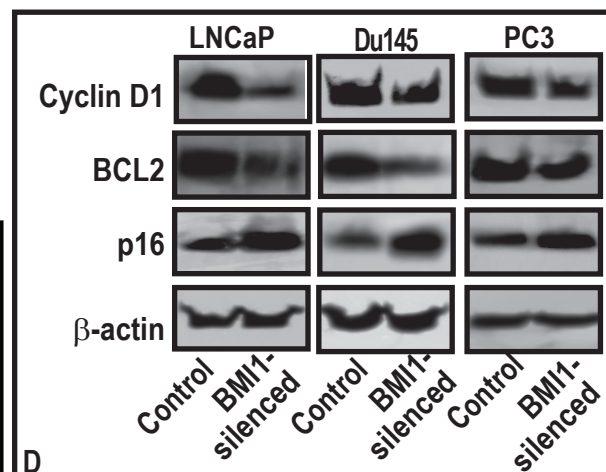
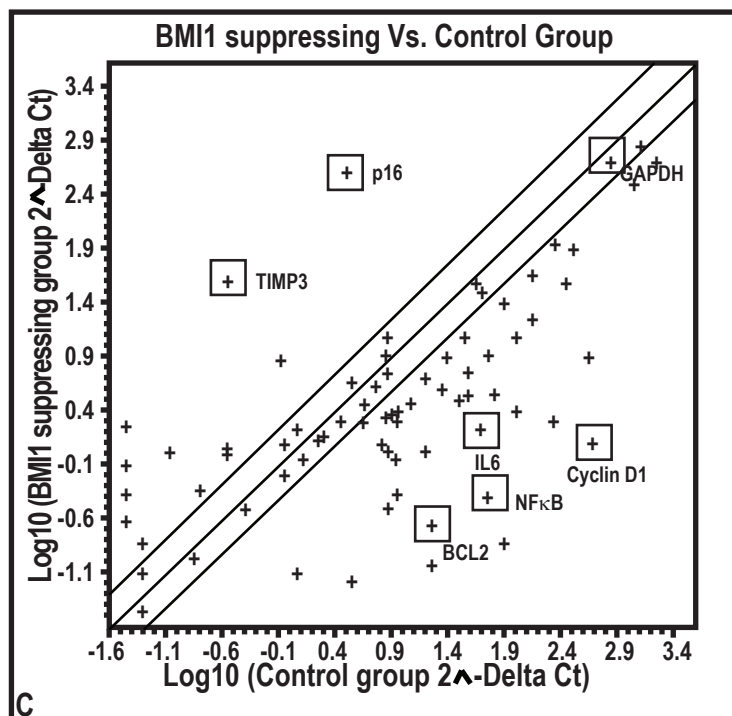
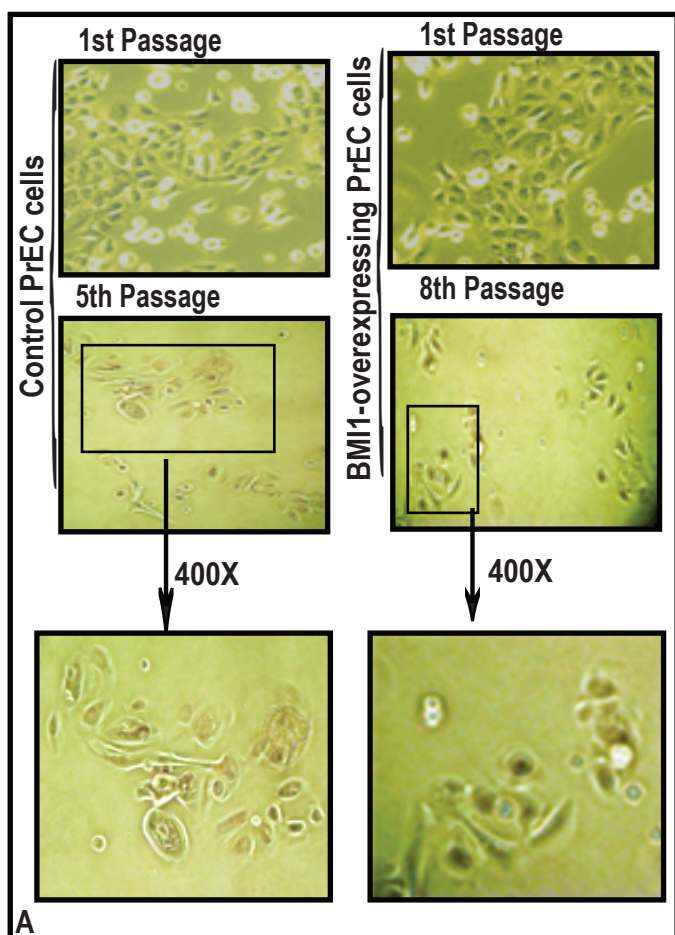


Figure 2 (A-H)

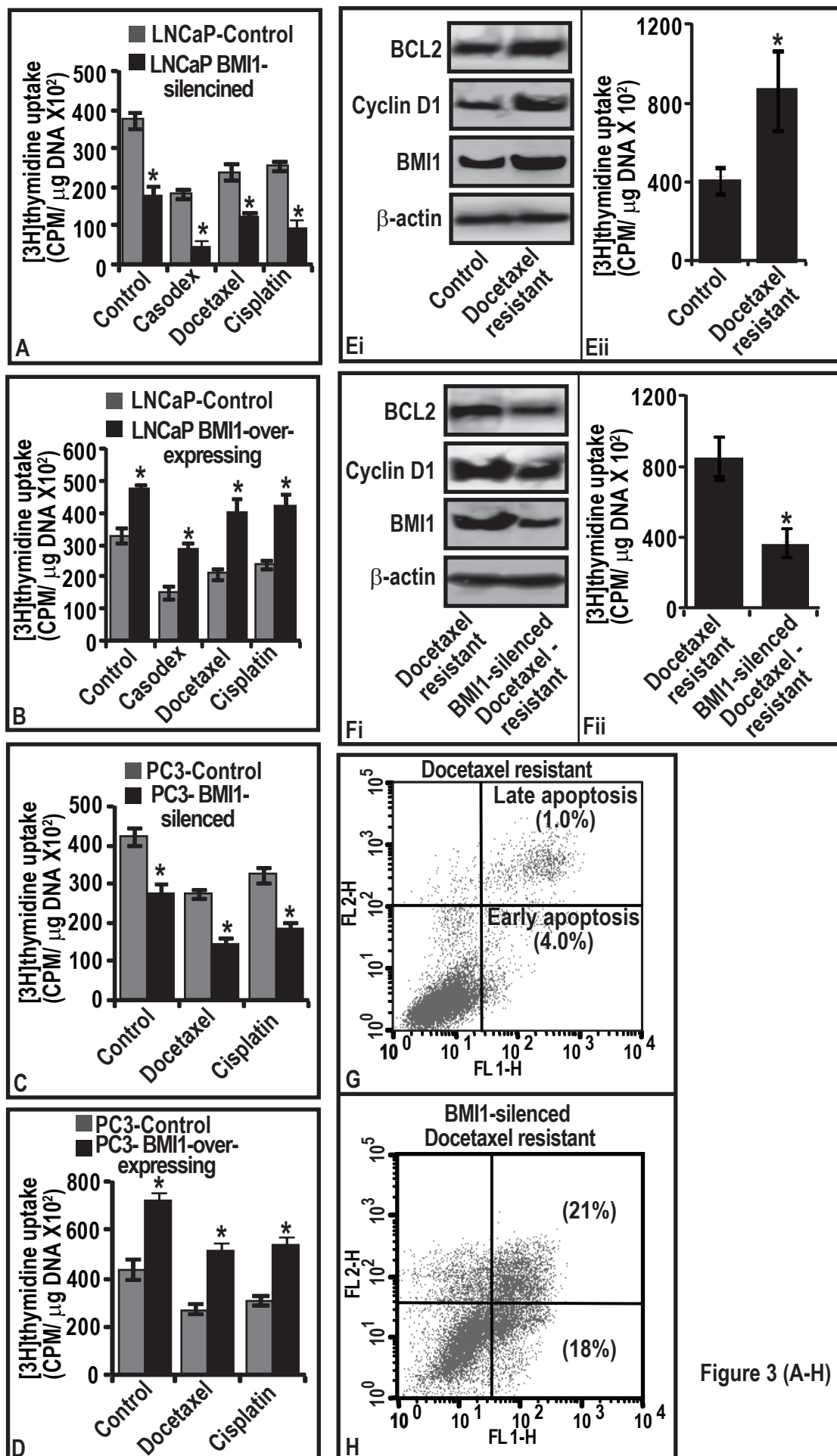


Figure 3 (A-H)

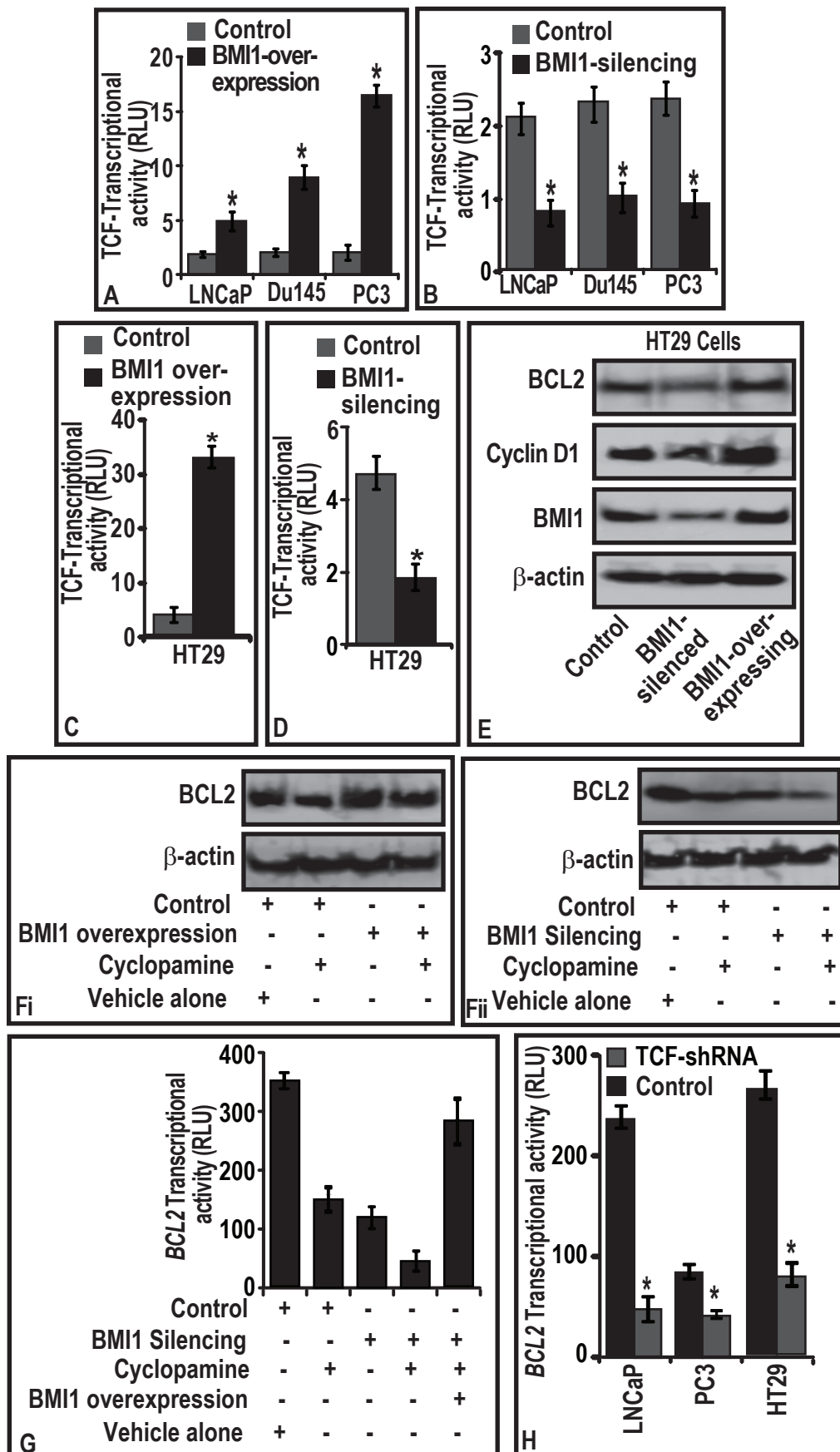


Figure 4 (A-H)

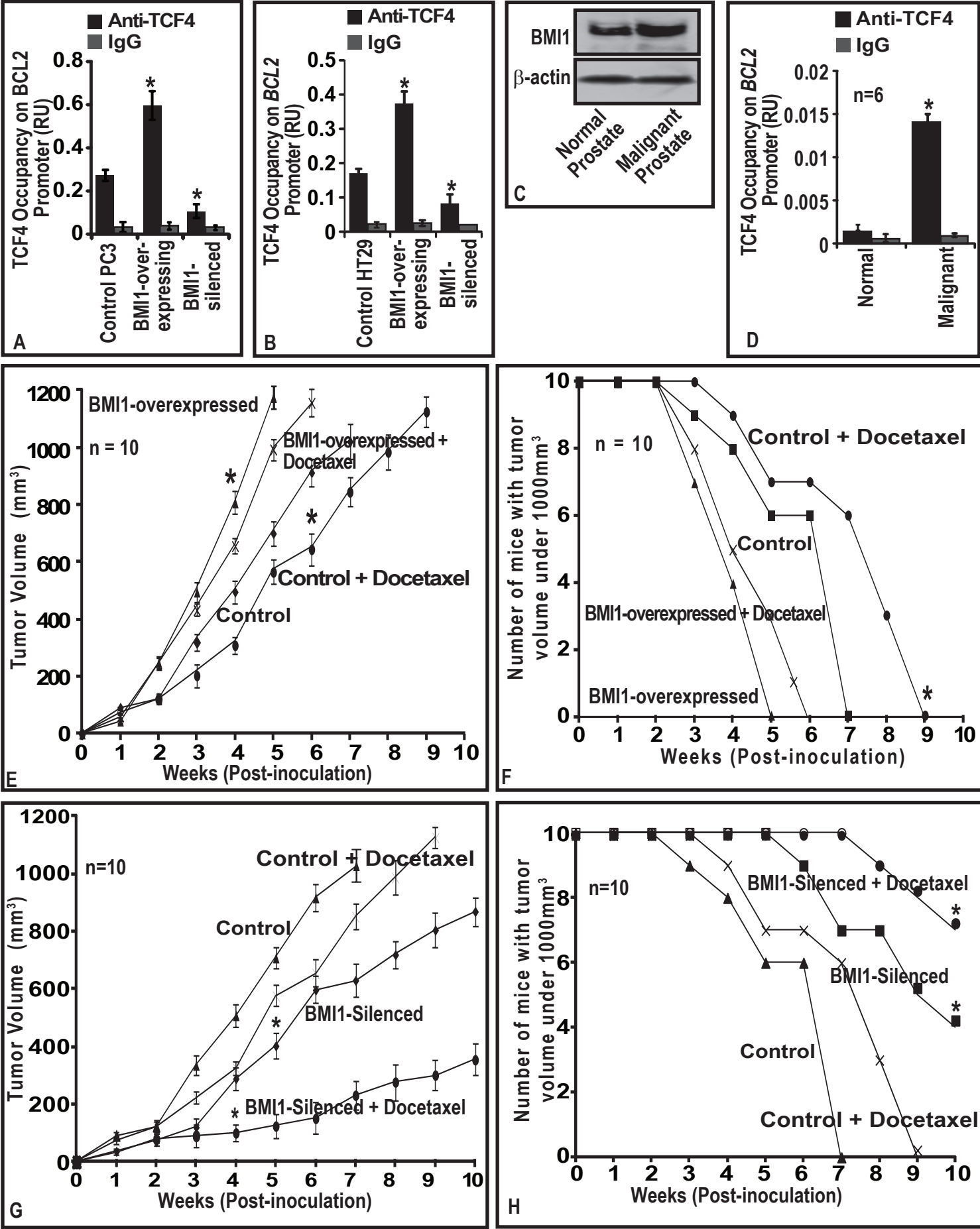
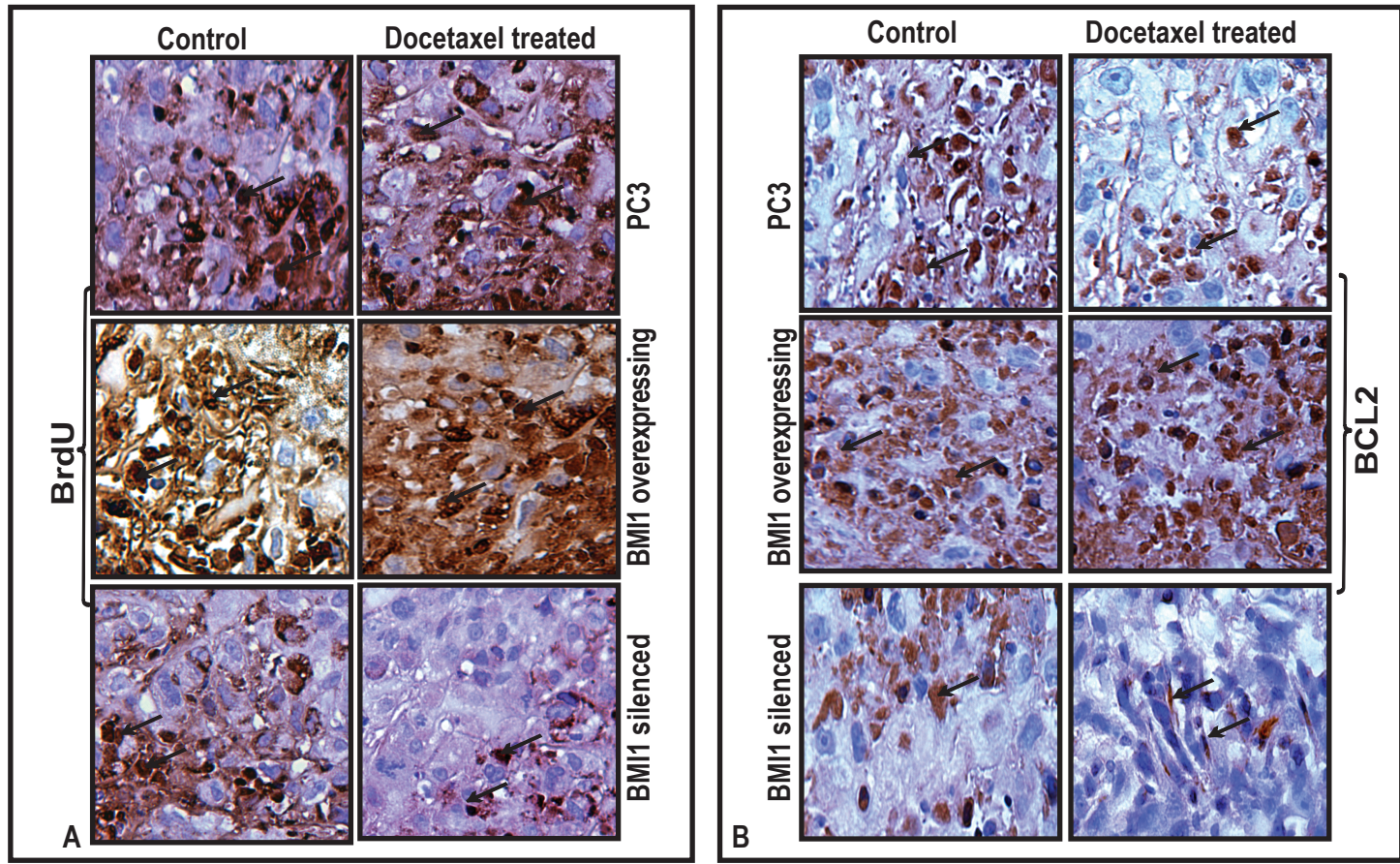


Figure 5 (A-H)

Figure 6



Supplementary Figure 1

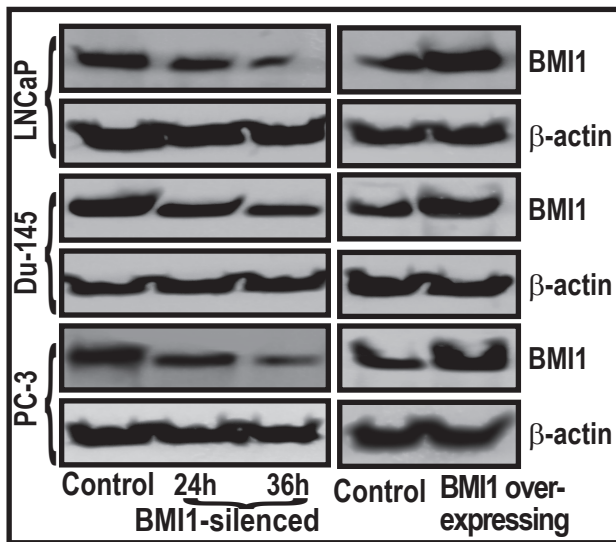


Figure 1

Supplementary Figure 2

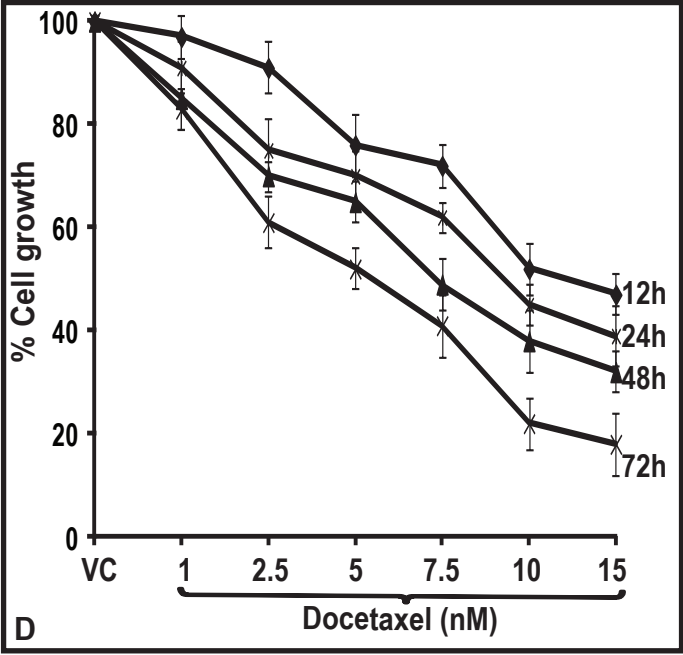
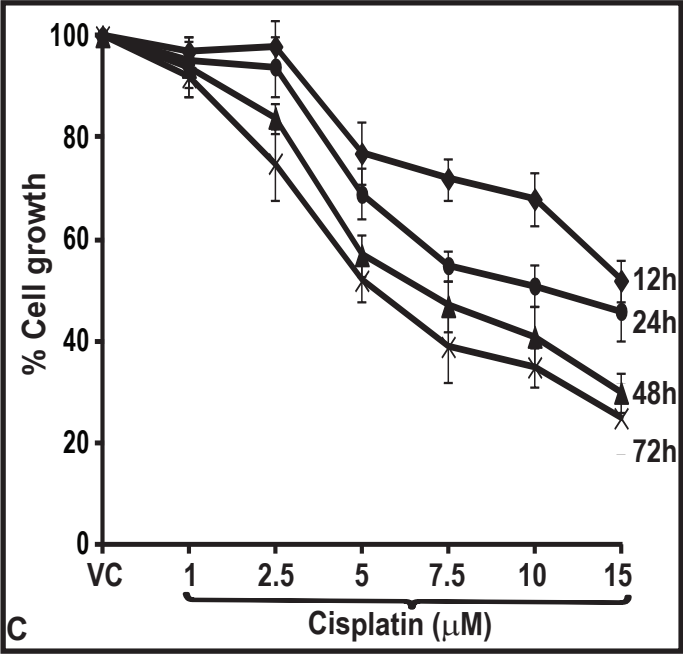
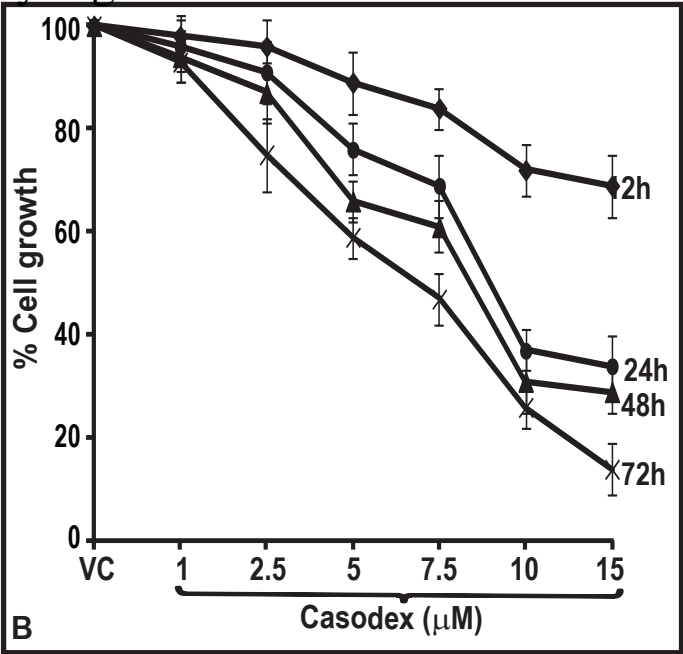
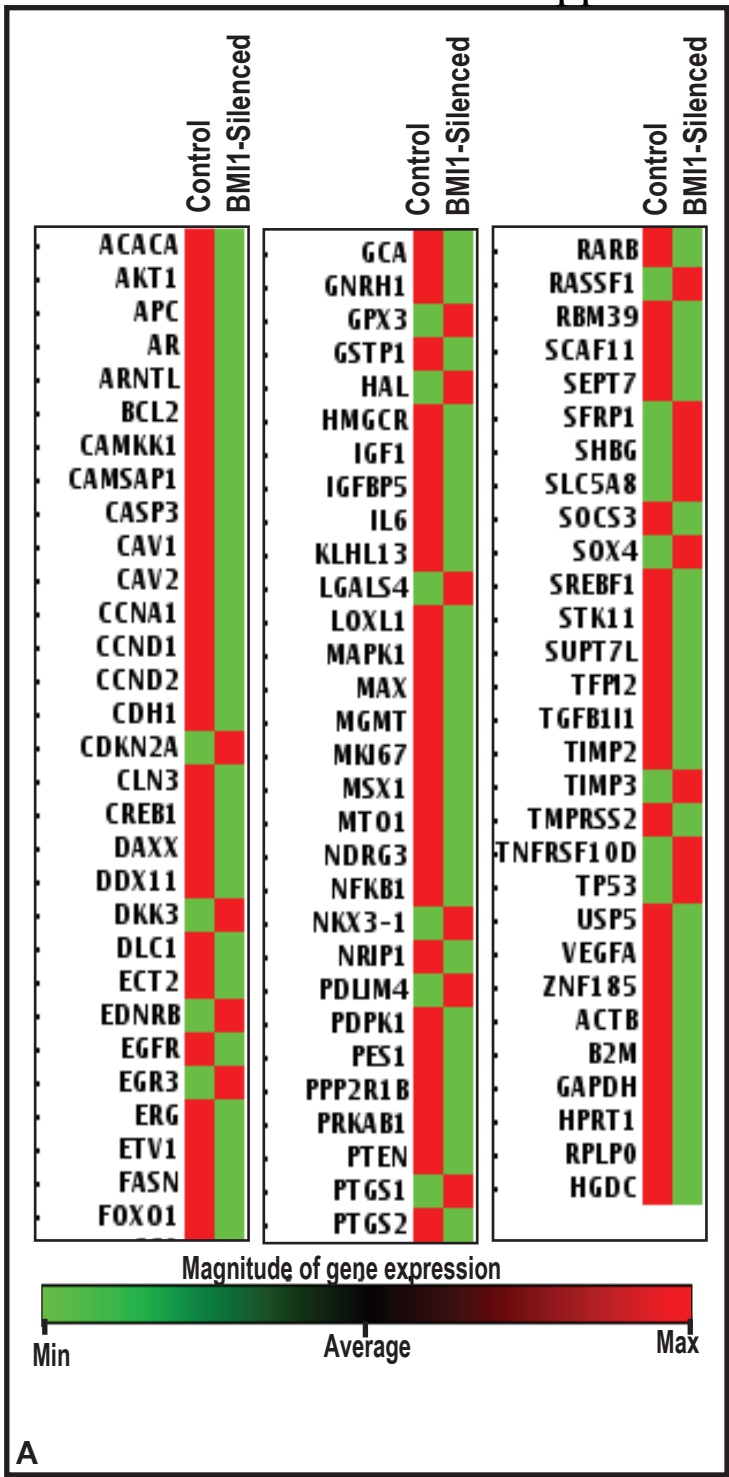


Figure 2(A-D)

Supplementary Figures 3 & 4

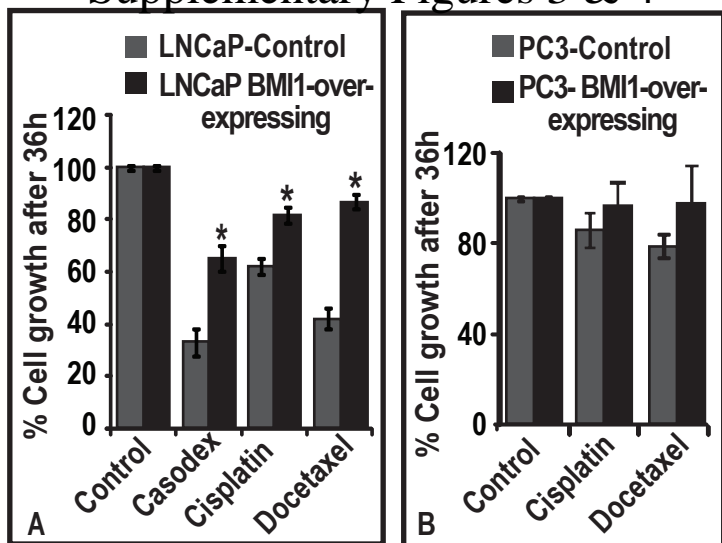
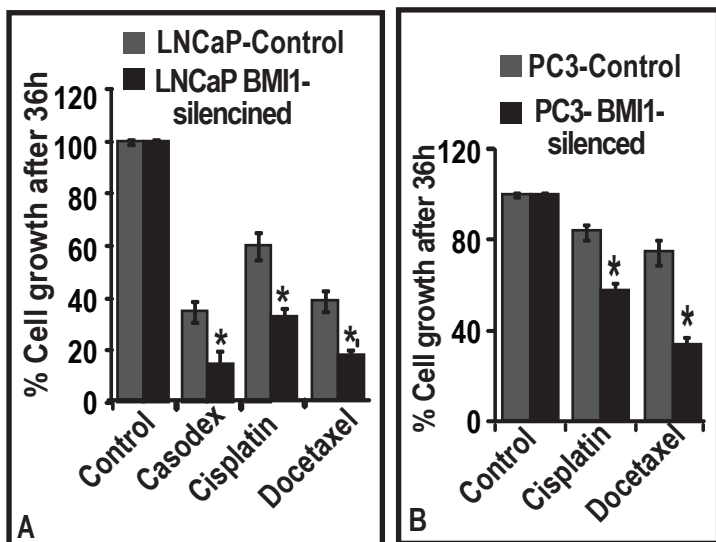


Figure 3A-B



Figures 4A-B

Acknowledgements of funding in manuscripts:

The funding (W81XWH-08-1-0605) from CDMRP-DOD has been acknowledged in the paper published. Published paper and a submitted report (for its publication in scientific journal) are attached as Appendices I-II.

- 1: Paper published in Scientific Journal. “ **Stem Cells**, 30: 372-78; 2012”. (**Appendix I**).
2. A part of our study funded by (W81XWH-08-1-0605) grant got accepted by for publication in **PLOS-ONE** journal. **Letter of acceptance from the journal** is attached as **Appendix II**. (*Note: Major part of study accepted for publication in PLOS-one journal was supported by institutional funds to Principal Investigator and collaborator*).
- 3: Paper Submitted to scientific journal “**Cell Death Differentiation**” for its publication and is under review.

Concise Review: Role of BMI1, a Stem Cell Factor, in Cancer Recurrence and Chemoresistance: Preclinical and Clinical Evidences

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Key Words. BMI1 • Cancer stem cells • Cancer recurrence • Chemoresistance • Cancer

ABSTRACT

There is increasing evidence that a variety of cancers arise from transformation of normal stem cells to cancer stem cells (CSCs). CSCs are thought to sustain cancer progression, invasion, metastasis, and recurrence after therapy. Reports suggest that CSCs are highly resistant to conventional therapy. Emerging evidences show that the chemoresistance of CSCs are in part due to the activation of B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), a stem cell factor, and a polycomb group family member. BMI1 is reported to regulate the proliferation activity of normal, stem, and progenitor cells. BMI1

plays a role in cell cycle, cell immortalization, and senescence. Numerous studies demonstrate that BMI1, which is upregulated in a variety of cancers, has a positive correlation with clinical grade/stage and poor prognosis. Although evidences are in support of the role of BMI1 as a factor in chemoresistance displayed by CSCs, its mechanism of action is not fully understood. In this review, we provide summary of evidences (with mechanism of action established) suggesting the significance of BMI1 in chemoresistance and recurrence of CSCs. *STEM CELLS* 2012; 30:372–378

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Traditional cancer therapies typically target the rapidly dividing tumor cells, however, some cells of the tumor are spared [1–3]. These spared tumor cells which are reported to be present within many tumor types exhibit the potential to regenerate and are called cancer stem cells (CSCs) [1–5]. This may explain the clinical scenario in which a tumor has an apparent volumetric reduction, however, is subsequently followed by local recurrence. While debate continues as to the precise identity and function of CSCs, there is general agreement that CSCs display increased chemoresistance and radioresistance [1–3, 6]. Therefore, understanding the biology of chemoresistance potential of CSCs may contribute to our understanding of tumor biology and would have far-reaching clinical implications. Although several molecules have been reported to confer chemoresistance to CSCs, much is not known whether stem cell factors play a role in chemoresistance of tumor cells including CSCs.

There is increasing evidence that polycomb group (PcG) proteins (discovered in *Drosophila* as epigenetic gene silencers) play a crucial role in cancer development and recurrence. PcG of proteins is composed of two multimeric protein complexes, that is, the polycomb repressive complex 1 (PRC1) and the polycomb repressive complex 2 (PRC2) [7]. The PRC1 complex includes B cell-specific Moloney murine

leukemia virus integration site 1 (BMI1), Mel-18, Mph1/Rae28, M33, Scmh1, and Ring 2, while the PRC2 complex includes Eed, EzH, Suz12, and YY1 [7]. BMI1 is reported to play an important role in self-renewal of stem cells and is associated with a number of human malignancies [2, 5, 8–10]. Recent studies suggest that BMI1 is involved in the initiation of cancer, and targeting BMI1 by gene therapy abolishes chemoresistance in tumor cells [2, 3]. In this review, we summarized (a) the evidences supporting the role of BMI1 in cancer recurrence and chemoresistance, (b) the mechanisms underlying, and (c) the potential approaches that could be used to target BMI1 for cancer therapy.

GENE AND PROTEIN STRUCTURE OF BMI1

Human *BMI1* gene localizes on short arm of chromosome 10 (10p11.23), which comprises 10 exons and 9 introns. The gene encodes a cDNA of approximately 3.4 kb length and a 36.8 kDa protein consisting of 326 amino acids, whereas mouse *Bmi1* gene encodes a protein of 45–47 kDa [2, 5]. With respect to amino acid sequence, a high degree of homology is found between human BMI1 and murine Bmi1 that was the first member of the PcG gene family identified in mammals. BMI1 protein contains a conserved ring finger domain in its N terminal end and a central helix-turn-helix-turn-

Author contributions: H.R.S: data collection, literature collection, and manuscript writing; M.S.: manuscript evaluation and literature verification and writing.

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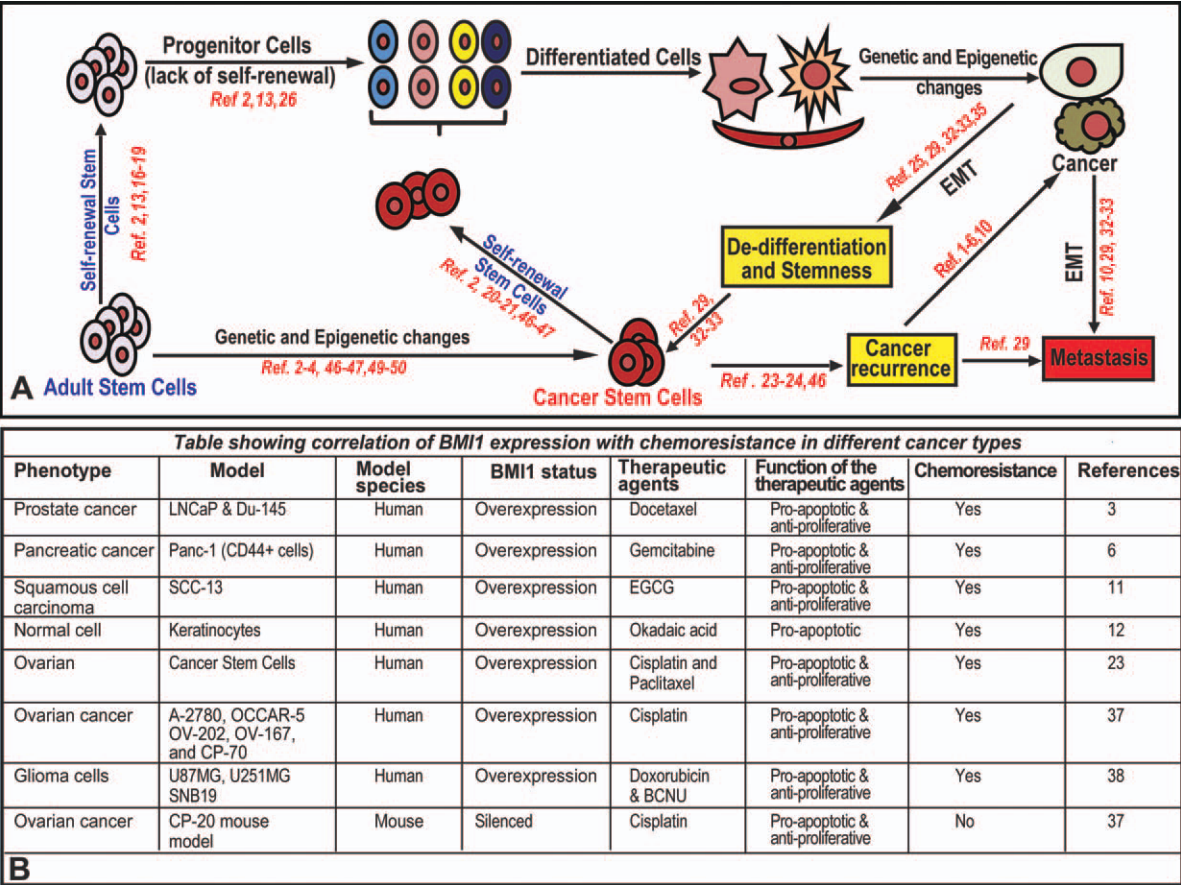


Figure 1. Role of BMI1 in malignant transformation of stem cells into cancer stem cells and chemoresistance. **(A):** Pictorial diagram representing role of BMI1 during cellular events associated with the malignant transformation of stem or differentiated cells into cancer stem cells. The numerical number given on each arrow within the figure represents the reference number cited in the manuscript. **(B):** Table showing correlation of BMI1 expression with chemoresistance in different cancer types assessed in in vitro and in vivo models. Abbreviations: BCNU, Bis-chloroethylnitrosourea; BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; EGCG, epigallocatechin-3-gallate; EMT, epithelial–mesenchymal transition.

helix-turn motif (H-T-H-T), which is essential for inducing telomerase activity [2, 5]. BMI1 contains two nuclear localization signals, KRRR and KRMK.

BMI1 has a ubiquitous pattern of expression in almost all tissues and its expression levels are observed to be high in the brain, esophagus, salivary gland, thymus, kidney, lungs, gonads, placenta, blood, and bone marrow [5]. Balasubramanian et al. [11] has reported the expression of BMI1 in basal and suprabasal keratinocytes. BMI1 is reported to be present in epidermal layers but not in dermis [12].

BMI1 IN NORMAL STEM CELLS

Stem cells are of two types (a) embryonic stem cells (ESCs) and (b) adult stem cells (ASCs). ESCs are pluripotent stem cells capable of developing into different cells, however, ASCs maintain and repair their resident tissues in adult organisms. Thus, self-renewal, differentiation, and prevention of senescence of ASCs are critical for tissue homeostasis. BMI1 plays crucial role for self-renewal and differentiation of leukemic stem and progenitor cells [13 and references therein]. BMI1 has also been reported to prevent senescence and immortalize cells through the activation of telomerase [8, 14]. It is reported that Bmi1 plays a crucial role during proliferation of normal stem and progenitor cells derived from fetal

liver [13]. Hosen et al. [15] showed that the expression of BMI1 is high in primitive hematopoietic stem cells (HSCs) and is decreased when HSCs are differentiated into a particular lineage. The self-renewal and maintenance of HSCs and neural stem cells (NSCs) were reported to depend on the level of BMI1 protein [8, 16]. These studies suggest a strong correlation of BMI1 with the differentiation and growth of stem cells [15, 16]. BMI1 is reported to play a crucial role during the self-renewal and maintenance of prostate, intestinal, lung epithelial and bronchioalveolar stem cells [17–19].

BMI1 AND CSCs

Over the past two decades, evidence has emerged to suggest that cancer could be considered as a stem cell disease and molecular mechanisms governing stem cell self-renewal are subverted during tumorigenesis to maintain cancerous growth (Fig. 1A, 1B) [2]. CSCs were first identified from the blood of patients with acute myeloid leukemia (AML) by Lapidot et al. in 1994 [4]. The CSC theory assumes that both primary and metastatic tumors develop from a small population of cancer cells possessing the characteristics of self-renewal and multipotency and are responsible for initiation and maintenance of tumors (Fig. 1A) [20, 21]. Additionally, CSCs can give rise to wide variety of differentiated cancer cells that comprise the

bulk of the tumor and provide the basis of tumor heterogeneity [20–22]. However, the stability of the CSC phenotype has not yet been completely understood [22]. Published reports suggest that CSCs are responsible for cancer recurrence after therapy and that this property of CSCs is attributed to the activation of different molecules including BMI1 [23–25].

BMI1 expression is frequently upregulated in various types of human cancers [1–2, 23–27]. There are reports that BMI1 acting as an epigenetic modifier protein is involved in the maintenance of CSCs [23, 25]. It is noteworthy that BMI1 is highly enriched in CSCs, however, all BMI1-expressing cells are not CSCs. BMI1 is coexpressed with other stem cell markers (CD133 and CD44) in CSCs [1, 6, 7, 23–26].

Aberrant BMI1 expression is reported in many CSC population. *Bmi1* has been reported to be highly expressed in CD133⁺ murine liver CSCs and play a role in maintenance of hepatic stem/progenitor cells [26]. Zhang et al. [23] observed that ovarian CSCs exhibit higher BMI1 levels than differentiated tumor cells. BMI1 has been shown to be involved in the regulation of CSCs from type-I neuroblastoma [9]. BMI1 was reported to regulate the self-renewal of CSCs by controlling their specific lineage commitment in an expression-dependent manner [9]. AML is a type of cancer in which the bone marrow makes abnormal myeloblasts, red blood cells, and platelets [13]. The proliferation of leukemic stem cells (LSCs) in a mouse model of AML was reported to be promoted by *Bmi1* [13]. *Bmi1*-expressing LSCs were able to induce leukemia when transplanted into irradiated mice, whereas *Bmi1*-null LSCs exhibited limited proliferative potential and were unable to induce disease [13]. This study suggested the critical role of *Bmi1* in proliferation of CSCs in leukemia [13]. Medulloblastoma is a type of brain tumor that originates from progenitor cells residing in the external cerebellum. Role of BMI1 in medulloblastoma can be ascertained from the fact that knock-down of *BMI1* in progenitor cells caused suppression in the proliferation and development of disease [27]. These studies suggest that the presence of BMI1 plays an important role in the proliferation of stem cells involved in tumorigenesis.

Different cell types that express BMI1 (such as endothelial cells, mesenchymal stem cells [MSCs], along with CSCs) reside within the tumor microenvironment [20, 28, 29]. The communication between CSCs and other cell types within tumor microenvironment plays an important role in invasion and therapeutic resistance [20, 28, 29]. Each established cell population within tumor exhibit a unique molecular marker that identifies and distinguishes it from other cell types [20–21, 28]. For example, MSCs express aldehyde dehydrogenase 1 (ALDH1) among breast CSCs population [20]. However, there is possibility that unique parental marker/trait still persists in cells that are in a stage of phenotypic transition such as mesenchymal transition [20, 28]. This also holds true with CSCs. A comprehensive discussion on this topic is beyond the scope of the theme of current manuscript.

BMI1, SELF-RENEWAL AND CELL CYCLE

BMI1 controls self-renewal and cell cycle by regulating the tumor suppressor proteins p16INK4a and p14ARF in cells [8, 14]. BMI1 has been shown to activate the self-renewal ability of NSCs [16]. Recently, Dong et al. [30] demonstrated that loss of BMI1 in endometrial cancer cells reduces expression of stemness genes SOX-2 and KLF4 suggesting that BMI1 is required for regulation of stemness of this cell type.

The p16INK4a protein inhibits binding of Cyclin D to CDK4/6, resulting in the (a) suppression of retinoblastoma

(RB) activity and (b) induction of cell cycle arrest [8, 31]. p19Arf (a homolog of human p14ARF) induces p53 and causes cell cycle arrest [8, 16, 31] (Fig. 2A, 2B). BMI1 promotes cell proliferation by suppressing p16INK4a/RB and/or p14ARF/MDM2/p53 tumor suppressor pathways [31]. The absence of BMI1 is reported to relieve the repression of the *INK4a* and resulting in the expression of p16INK4a and p14ARF. Data accumulated so far suggest that BMI1 abolishes cell cycle check points p16/p14 in various cell types (which exhibit different rates of growth/cell cycle kinetics) [7]. We speculate that this holds true for CSCs too. However, the possibility is that BMI1 could not be a sole factor deciding the fate of cells. Although BMI1 is present in CSCs, there is possibility that different subpopulation among CSCs (such as quiescent CSCs) exhibit different rate of growth. This could be possible due to the presence of factors other than BMI1 [18].

BMI1, EPITHELIAL–MESENCHYMAL TRANSITION AND CSCs

The epithelial–mesenchymal transition (EMT) is a key developmental program that is often activated during cancer development [32, 33]. The occurrence of EMT in cancer cells may lead to the number of changes including loss of polarity and epithelial cell markers, loss of contact inhibition, reorganization of the actin cytoskeleton, remodeling of extracellular matrix components, gain of mesenchymal phenotypes along with genetic/epigenetic modifications of different genes, and persistent activation of different growth factors [32, 33]. Published reports suggest a direct link between the EMT and the gain of MSC-like properties [32, 33]. Raimondi et al. [33] reported that the induction of EMT program does not only allow cancer cells to disseminate from the primary tumor but also promotes their self-renewal capability. The sustained stimulation of growth factors may result in an upregulation of diverse gene products in CSCs and their differentiated progenies during the EMT process [32, 33]. Experimental evidence revealed that EMT is involved in anticancer drug resistance [32]. Thus, identification of molecular events that regulate EMT could lead to the development of a new therapeutic approach to suppress growth of CSCs. Song et al. [25] demonstrated that ectopic expression of BMI1 in normal nasopharyngeal epithelial cells is sufficient to cause EMT. Furthermore, this study showed that BMI1 induces EMT by targeting the tumor suppressor PTEN [25]. This in vitro observation was consistent with a cohort of human biopsy samples where an inverse correlation between BMI1 and PTEN was observed [25]. Recently, Yang et al. [29] showed that BMI1 is essential for EMT during tumor development in head and neck cancer patients. This study showed that increased levels of BMI1 were correlated with the worst prognosis in patients with head and neck cancer [29]. The molecules which are frequently altered in cancer cells during the EMT process are E-cadherin, N-cadherin, Vimentin, Tenascin C, NF- κ B, SLUG, TWIST, SNAIL, β -Catenin, and CXCR4 [32, 33]. Collectively, these molecules are thought to contribute to the metastatic phenotypes of CSCs and enhance resistance to radiotherapy and chemotherapy [32, 33]. It has been reported that normal human mammary epithelial cells adopt a mesenchymal phenotype and exhibit stem cell-like properties upon expression of SNAIL and TWIST [32]. TWIST is reported to inhibit the senescence inducer proteins (p16 and p21) and co-operates with activated rat sarcoma (RAS) to trigger EMT [32]. Induction of SLUG is known to suppress E-cadherin, which results in the promotion of EMT [34]. Interestingly, CD133⁺ breast

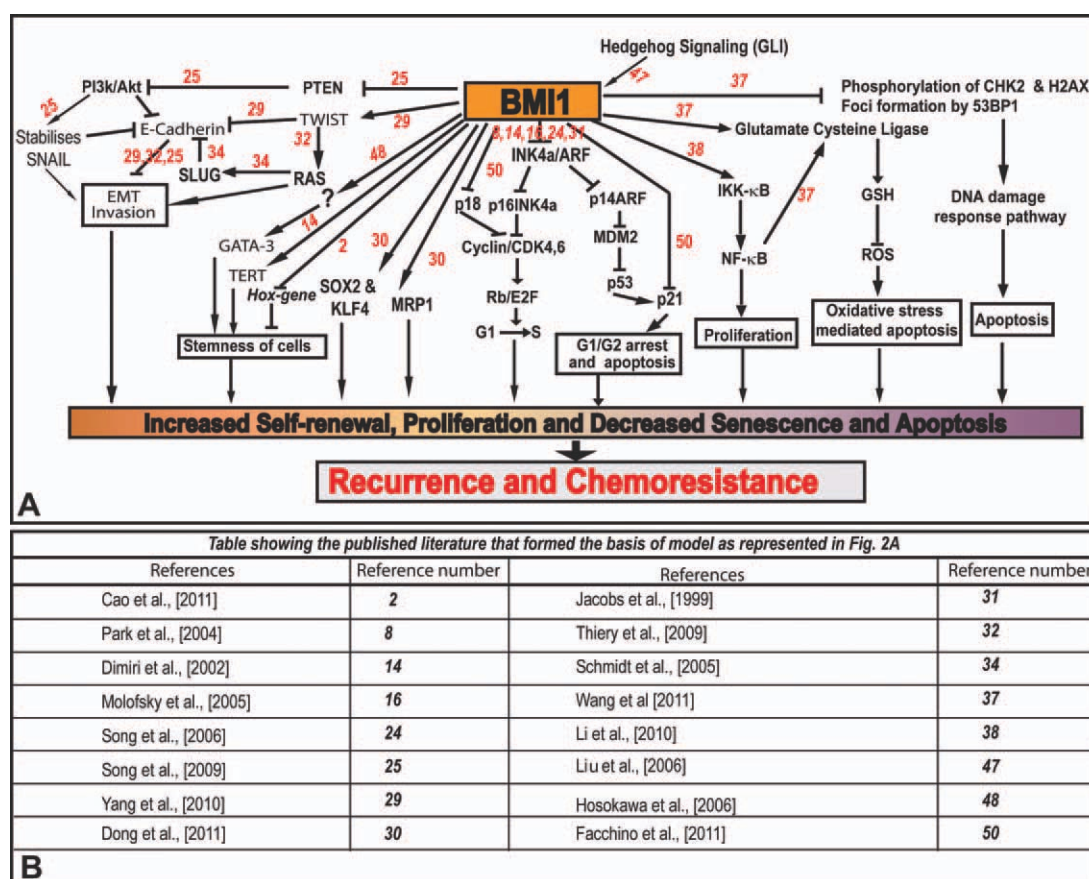


Figure 2. Role of BMI1 in cancer recurrence and chemoresistance. (A): Flowchart represents role of BMI1 and its interacting proteins during self-renewal, proliferation, and chemoresistance of cancer cells. (B): Table showing the published literature that formed the basis of model as represented in (A). The numerical number given on each arrow within the figure represents the reference number cited in the manuscript. —|— represents inhibition and —→ represents activation. Abbreviations: BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; EMT, epithelial–mesenchymal transition; GSH, reduced glutathione; IKK, IKB kinase; NF, nuclear factor; ROS, reactive oxygen species.

CSCs that express SLUG are also found to express high BMI1 [35]. BMI1 in co-operation with TWIST1 was reported to promote cancer dedifferentiation and metastasis [29]. Keeping in view that (a) EMT and stemness are interlinked processes, (b) EMT and stemness processes confer chemoresistance to tumor cells, and (c) BMI1 plays role in both EMT and stemness processes, the importance of BMI1 in chemoresistance as a major factor is further strengthened.

BMI1 AND CHEMORESISTANCE: PRECLINICAL EVIDENCES

The inability of tumor cells to undergo apoptosis in response to chemotherapy poses a selective advantage for tumor progression, metastasis, and resistance to therapy. BMI1 has been reported to be associated with the protection of tumor cells from apoptosis (Fig. 1B). Cui et al. [9] showed that the ectopic expression of BMI1 rescues keratinocytes from stress-induced apoptosis. Bmi1 knockdown was observed to increase the apoptosis in lymphocytes in spleen and thymus in an animal model [36]. Zhang et al. [23] observed that ovarian CSCs exhibiting high BMI1 levels have increased resistance to Cisplatin and Paclitaxel. Crea et al. showed that BMI1 silencing significantly enhanced the antitumor efficiency of Docetaxel against prostate cancer cells. BMI1 (by modulating antioxidant machinery) was observed to allow prostate tumor cells to survive after chemo-

therapy [3]. Examination of clinical datasets revealed a positive correlation of BMI1 and antioxidant gene expression in patients exhibiting chemoresistance [3]. Recently, Wang et al. [37] reported that BMI1 is involved in chemoresistance of ovarian cancer cells, and targeting BMI1 by gene therapy sensitizes tumor cells to Cisplatin chemotherapy. Modulation of reduced glutathione (GSH) and CHK2 and H2AX molecules by BMI1 was reported as the underlying mechanism for chemoresistant behavior of ovarian tumor cells [37]. BMI1 silencing was found to reduce intracellular GSH levels and sensitize cancer cells to Cisplatin [37]. It is noteworthy that Cisplatin-induced apoptosis in such cell was found to be mediated by reactive oxygen species (ROS) generation [37]. Recent studies showed that overexpression of BMI1 rescues tumor cells from the apoptosis induced by Okadaic acid and Epigallocatechin-3-gallate, well-known apoptotic agents [11, 12]. Interestingly, artificial introduction of BMI1 in chemosensitive tumor cells was observed to confer chemoresistance in such cells [11]. Yin et al. [6] showed that CD44⁺/CD24⁺ pancreatic cancer cells expressing high levels of BMI1 exhibit chemoresistance to Gemcitabine treatment. Li et al. [38] reported that BMI1 by activating NF-κB significantly inhibits Doxorubicin-, BCNU-, and UV irradiation-induced apoptosis in glioma cells. Recently, we observed that the reduction of BMI1 protein levels by gene therapy abolishes chemoresistance in prostate carcinoma cells (Siddique et al., unpublished data). Taken together, these studies support the role BMI1 plays in conferring chemoresistance to tumor cells.

BMI1 AND CHEMORESISTANCE: CLINICAL EVIDENCES

The clinical significance of BMI1 in chemoresistance and its correlation with therapy failure in several cancer types has been established [5, 9, 10, 39–40]. BMI1 was found to be one of the key regulatory factors determining a cellular phenotype captured by the expression of a death-form-cancer signature in a broad spectrum of therapy-resistant cancers, including five epithelial (prostate, breast, lung, ovarian, and bladder cancers) and five nonepithelial (lymphoma, mesothelioma, medulloblastoma, glioma, and AML) malignancies [39]. Glinsky et al. [39] described a conserved BMI1-driven pathway of 11-gene signature which defines stemness of highly invasive tumors of multiple tissue origin and correlation with therapy failure. High level of BMI1 in tumors was reported to be positively correlated with poor prognosis in nasopharyngeal carcinoma patients [24]. BMI1 was identified as predictive factor for overall survival in patients with head and neck squamous cell carcinomas (HNSCC) [41]. BMI1 levels were observed to be increased in 79% of HNSCC patients, and a positive correlation was observed between BMI1 levels and lack of response to radiotherapy or chemotherapy [41]. Van Kemenade et al. [42] reported that poor outcome and aggressive tumor behavior were correlated with high BMI1 levels in patients with non-Hodgkin B-cell lymphomas and nasal pharyngeal carcinoma. Li et al. [38] showed that BMI1 was upregulated in 93.9% glioma specimens from 297 patients. This study showed that BMI1 expression was inversely correlated with survival time of glioma patients and positively correlated with the poor prognosis of the disease [38]. Mihic-Probst et al. [10] studying 329 melanoma patients reported that high expression of BMI1 in 64% of primary and 71% metastatic melanoma was associated with clinical progress of the disease. Recent reports show a correlation between BMI1 levels and recurrence cum survival of disease in tongue cancer, oropharyngeal squamous cell cancer, and non-small cell lung cancer (NSCLC) patients [5, 43, 44]. Disease-free survival for stage I and II of NSCLC patients who had received adjuvant therapy was reported to be better in BMI1-negative patients than BMI1-positive counterparts [44]. We observed a stage-dependent increase in human prostatic tumors and decreased chemoresistance in cells exhibiting reduced BMI1 levels (Siddique et al., unpublished data). Collectively these studies also suggest that BMI1 might be applicable as predictive markers of therapy during the follow-up of patients undergoing chemotherapy.

MOLECULAR MECHANISMS OF BMI1-INDUCED CHEMORESISTANCE

Chemoresistance has been reported to be caused by the aberration of several molecular pathways in tumor cells. CSCs have been shown to display chemoresistance through (a) modulation of DNA repair machinery, (b) ATP-binding cassette (ABC) multidrug resistance, (c) quiescence, and (d) upregulation of antiapoptotic genes [45]. Emerging evidences support the notion that BMI1 is an important molecule in the process of chemoresistance. However, the precise mechanism of BMI1 on the regulation of chemoresistance in tumor cells is not completely understood. As presented in Figure 2, BMI1 is reported to modulate several molecular pathways within the cells. BMI1 has been shown to induce its effect at epigenetic as well as genetic level [7, 13, 46]. It is believed that chroma-

tin modifications induced by PcG proteins (including BMI1) create an obstacle to transcription factors and RNA polymerase binding [46]. BMI1 has been shown to modulate chromatin by (a) forming a complex with methylated Lys₂₇ of H3 and (b) catalyzing the ubiquitinylation of histone H2A [7, 46]. The co-operation between the Eed complex (that modifies chromatin by recruiting histone deacetylases) and BMI1 complex leads to the silencing of target gene expression [7, 46]. BMI1 induces immortalization of cells by downregulating the p16INK4a and p14ARF [8, 16]. Huber et al. [5] reported a correlation between low expression of p16 and high expression of BMI1 in human cancer patients. It is reported that the cooperation of BMI1 with c-MYC results in induction of telomerase activity and downregulation of *INK4a*/ARF [36].

Sonic Hedgehog (SHH) pathway is reported to play a role in the self-renewal of breast stem/progenitor cells [47]. SHH-activated mammosphere formation is reported to be mediated by BMI1 [47]. BMI1 is reported to regulate intracellular GSH levels by modulating glutamate cystine ligase, which is also positively regulated by Nrf-1 and nuclear factor κ B (NF- κ B) [37]. It is noteworthy that BMI1 expression was reported to be positively associated with activity of Nrf-1 and NF- κ B in glioma cells [38]. BMI1 is reported to occupy the *PTEN* locus and downregulates *PTEN* expression [25]. Occupancy of BMI1 on *PTEN* locus results in the activation of Phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathway, stabilization of SNAIL, and downregulation of E-cadherin. BMI1 directly occupies the promoters of CDH1 (which encodes E-cadherin) and *INK4a* [25]. Lee et al. [12] showed that BMI1 influences cell proliferation by increasing the expression levels of cyclin-dependent Kinase 2, 4 (CDK2, CDK4), and Cyclin D1. BMI1 is reported to regulate stability of GATA binding protein 3 (GATA3), a transcription factor that is involved in Th2 cell development and differentiation [48]. Recently, Dong et al. [30] demonstrated that loss of BMI1 in endometrial cancer cells reduces expression of drug resistance gene MRP1, suggesting that BMI1 is required for the drug resistance. Quiescent nature of CSCs represents an inherent mechanism that at least partially explains chemotherapy resistance and recurrence in post-therapy in cancer patients [18, 20, 30]. Recent study by Tian et al. [18] suggest that Bmi1 plays an important role in the maintenance and growth of quiescent cells. Bmi1-expressing quiescent cells were shown to contribute to the generation of epithelial cells of intestine [18]. It is noteworthy that this effect of BMI1 was observed under conditions when proliferative cells were not sufficient and BMI1 expressing-quiescent cells were found to grow into tissue [18].

BMI1: A POTENTIAL TARGET FOR CANCER THERAPY

CSCs may be eliminated by selectively targeted therapies against BMI1 [49, 50] (Siddique et al., unpublished data). However, it would be much complex to selectively target CSCs without any harmful effects to normal stem cells because normal stem cells and CSCs share the same pathways to maintain their self-renewal capability. It appears that CSCs are more likely to be more dependent on certain putative pathways. In this context, Liu et al. showed that human BMI1 is critical for the short-term survival of cancer cells, and inhibition of BMI1 has minimal effect on the survival of normal cells. These findings provide a foundation for developing a cancer-specific therapy targeting BMI1 [49]. Recently, Facchino et al. showed that glioblastoma multiforme (GBM) stem cells acquire an oncogenic trait by BMI1 overexpression thus distinguishing CSCs from normal stem cells. This situation was observed to render GBM stem cells

more sensitive to BMI1 inhibition than normal stem cells [50]. Based on compelling evidences (which suggest the critical role of BMI1 in growth and proliferation), using BMI1 as a target for anticancer therapy seems an ideal option. Wang et al. successfully tested 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine nanoparticles carrying small inhibitory RNA (siRNA) to target BMI1 and reported an inhibition in the growth of chemoresistant ovarian tumors implanted in a xenograft mouse model [37]. This study showed that gene therapy-induced BMI1 silencing along with Cisplatin completely abrogated ovarian tumor growth [37].

We recently showed that targeted inhibition of BMI1 by adopting gene therapy approach resulted in the reduction in the invasive potential and tumorigenic potential of prostate cancer cells (Siddique et al., unpublished data). We have embarked upon a broad program aimed to evaluate the potential and usefulness of BMI1 as a molecular target for human cancers. We have developed specific BMI1 small molecule inhibitors (Siddique et al., unpublished data), which were observed to inhibit the proliferative potential of prostate, pancreatic and skin cancer cells (Siddique et al., unpublished data).

CONCLUSIONS

BMI1 has been reported to be associated with the progression, recurrence, and chemoresistance to the various types of cancer

cells. Hence, it is of great clinical value to further understand the molecular mechanism underlying the regulation of BMI1 in CSCs and chemoresistance. This will not only help in understanding the role of BMI1 in the growth of CSCs and chemoresistance but will also provide insights for the establishment of new strategies and effective clinical therapies for the treatment of chemoresistant cancers. Taken together, these studies show that BMI1 has the potential to be developed as a target for therapeutic agents and small molecules efficiently targeting BMI1 offer an option as future anticancer drugs.

ACKNOWLEDGMENTS

The data from our laboratory cited in this manuscript was funded in part by a New Investigator grant from CDMRP-Department of Defense (W81XWH-08-1-0605) to the corresponding author.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflict of interest.

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APPENDIX II

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1) On page 2 (Abstract), please delete the last sentence of the "Results" subsection in parenthesis (.. distinguishing it from PSA..).

2) On page 15, "discussion section", 4th line from top, the sentence should be re-written as follows: "PSA is androgen-dependent and its expression is regulated by androgen receptor [20]".

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